Plant Defence in a Tritrophic Context

Chemical and behavioural analyses of the interactions between spider mites, predatory mites and various plant species

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Proefschrift
ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit
prof. dr. ir. L. Speelman
in het openbaar te verdedigen
op vrijdag 25 april 2003
des namiddags te half twee in de Aula

Chemists may be annoyed by variation, while ecologists are intrigued by it.
L. Vet, 1999. J. Chem. Ecol. 25: 31-49.

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Plant Defence in a Tritrophic Context: Chemical and behavioural analyses of the interactions between spider mites, predatory mites and various plant species
Thesis Wageningen University

ISBN 90-5808-806-5

Stellingen

1. Plantensoorten met een goede directe verdediging tegen spintmijten, maken in veel gevallen toch gebruik van indirecte verdediging.
   Dit proefschrift.

2. Planten die zijn aangetast door spintmijten produceren in veel gevallen de verbinding methylsalicylaat. Echter, methylsalicylaat alleen is niet specifiek genoeg om te kunnen dienen als indicator voor spintmijt aantasting.
   Dit proefschrift.

3. Gebruik van synthetische mengsels om natuurlijke mengsels na te bootsen kan tot verkeerde conclusies leiden als deze getest worden op biologische activiteit.
   Turlings et al., 1991. J. Chem. Ecol. 17: 2235-2251.

4. Subtractieve combinatie is een betere methode om synergisme tussen twee of meer stoffen aan te tonen dan additieve combinatie.
   Byers, 1992. J. Chem. Ecol. 18: 1603-1621.

5 Solid Phase Microextration (SPME) is geen goede methode om inzicht te verkrijgen in de juiste natuurlijke verhoudingen van een mengsel van stoffen.
   Monnin et al., 1998. J. Chem. Ecol. 24: 473-490; Agelopoulos and Pickett, 1998. J. Chem. Ecol. 24: 1161-1172.

6. De burger vindt 'Maatschappelijk Verantwoord Ondernemen' erg belangrijk, maar als consument wil hij hiervoor niet betalen.
   Dutilh et al., 2003. VMT 3: 19-21.

7. Ondanks alle keurmerken op producten, is de consument nog steeds het slachtoffer van selectieve publieksvoorlichting.

8. 'Chemisch is gevaarlijk en natuurlijk is gezond' is een misvatting.

Stellingen behorend bij het proefschrift:

Plant Defence in a Tritrophic Context:
Chemical and behavioural analyses of the interactions between spider mites, predatory mites and various plant species

Wageningen, 25 april 2003 Cindy E. M. van den Boom

Voor mijn ouders

Voorwoord

Tijdens mijn promotie onderzoek heb ik gedurende vier jaar gewerkt aan interacties die plaatsvinden tussen 3 verschillende niveaus van een voedselweb. In mijn werkzaamheden bestonden dergelijke interacties ook tussen de groepen waar ik mijn onderzoek heb uitgevoerd. Dit waren de groep fytochemie (chemie van plantaardige stoffen), de ondersteunende groep analytische chemie (analyseren van chemische stoffen) behorende bij de leerstoelgroep Bio-organische Chemie en de leerstoelgroep Entomologie (insectenkunde). Door de steun van verscheidene mensen uit deze drie groepen is dit proefschrift tot stand gekomen.

Binnen de groep fytochemie kwam ik te werken onder de stimulerende leiding van Teris van Beek. Ik kreeg de mogelijkheid om onder zijn toezicht tot mijn einddoel te kunnen komen. Tot op het laatste moment heb ik prettig met hem samengewerkt. Mijn promotor Aede de Groot heeft zich op enige afstand uitstekend op de hoogte gehouden van mijn voortgang. Binnen de leerstoelgroep Organische Chemie stond hij altijd open voor discussie over allerlei alledaagse labaangelegenheden. Bij de groep analytische chemie werd ik ondersteund door Maarten Posthumus, om met behulp van gas chromatografie in combinatie met massa spectrometrie de belangrijke plantenstoffen te kunnen analyseren. Hem wil ik nadrukkelijk bedanken voor de hulp die hij me hierbij gegeven heeft. Hiernaast heeft hij me bovendien nog enthousiast weten te maken voor de sterrenkunde. Bij de leerstoelgroep Entomologie zorgde mijn promotor Marcel Dicke voor een goede en enthousiaste begeleiding. Zijn kritische oog brachten mijn artikelen op een hoger niveau.

Bij deze wil ik al mijn begeleiders nadrukkelijk bedanken voor alles wat ze voor me gedaan hebben en klaar te staan indien het nodig was.

During the last few months of the practical research work I received help from a foreign student named Damien Raingeard. He obtained good results on the fractionation system. I thank him very much for his support and good discussions.

Bij Organische chemie hebben Marijke, Elbert, Ronald, Pleun, Elly, Gabriëlle en Ineke allen een bijdrage geleverd aan het tot stand komen van mijn proefschrift. Ter ondersteuning van mijn experimenten wil ik Herman Dijkman (Entomologie) bedanken voor het onderhouden van de roofmijtenkweek en Henk Smid en Pieter Kuiken voor hun goede zorgen voor de planten die in de kassen van Unifarm opgekweekt werden.

Tevens wil ik van organische chemie alle AIO's bedanken voor een leuke tijd met AIO-reizen naar Zweden en de Verenigde Staten, filmavondjes, zwemavonden en gewoon lekker koffie drinken. Ook bij entomologie werden er regelmatig culturele avonden georganiseerd en gingen we vaak met een grote groep schaatsen. Van de AIO's wil ik vooral Frederique en Jetske bedanken voor hun ondersteuning in de periode dat ik in Den Haag werkte. Hun enthousiasme om artikelen op te zoeken en naar mij op te sturen, heb ik zeer gewaardeerd. Yvonne, jij stond altijd klaar als ik een slaapplaats nodig had, als ik even mijn frustraties af wilde reageren en om mijn leesversie in te leveren. Vele malen dank hiervoor!! Hugo, naast een collega werd jij ook een goede vriend om mee te zeilen als we niet met het mooie weer op het lab wilden zitten. Achter op de motor gingen we samen naar het zuiden van België om naar een schitterende zonsverduistering te kijken. Met Remco, Jetske en Eric (E) heb ik vele avonden in de Vlaam doorgebracht om elkaar van onze wetenschappelijke avonturen op de hoogte te houden, soms tot vervelens toe voor E. Hier kwamen vaak erg creatieve stellingen tot stand.

Naast mijn promotieonderzoek heb ik getracht om samen met Jan, Corien, Eyke, Marijke en Marc in het Wageningse AIO- en OIO-overleg voor een aantal verbeteringen te zorgen voor de AIO's en OIO's op de universiteit. Tot mijn verbazing hebben we het uiteindelijk toch voor elkaar gekregen, dat proefschriften op de webside van de Wageningen Universiteit geplaatst kunnen worden en dat de bibliotheek tegenwoordig met minder proefschriften genoegen neemt. Het was altijd erg gezellig tijdens onze efficiënte vergaderingen. Wanneer gaan we trouwens naar de Bahama's?

Niet te vergeten wil ik al mijn goede vrienden bedanken voor hun onafgebroken steun en toeverlaat tijdens mijn schrijfperiode. Een aantal vrienden belden regelmatig om mijn vorderingen te controleren en tevens gezellig bij te kletsen. Hiervan wil ik in het speciaal Jacques, Marijke, Pieter, Erwin, Remi en Erik L. bedanken voor hun regelmatige telefonische ondersteuning. Met name Jacques en Marijke hebben vele malen voor de broodnodige ontspanning gezorgd en hebben me hierdoor behoed voor veel onnodige proefschrift stress. In het bijzonder wil ik mijn huisgenoten bedanken voor alle gezelligheid.

Bij Stichting Milieukeur ben ik mijn collega's dankbaar voor hun support, ook al vroegen ze zich vaak af wat ik in godsnaam aan het doen was. In de avonduren kon ik me bij de Atletiekvereniging 'De Haag' uitleven om hierna weer achter de computer te kunnen kruipen. In het bijzonder wil ik Judith bedanken voor haar creatieve bijdrage voor het tot stand komen van de omslag.

Zonder mijn beide paranimfen Matthew en Remco, was dit proefschrift waarschijnlijk niet tot stand gekomen. Allebei wil ik jullie hartelijk danken voor jullie onvoorwaardelijke steun wanneer het in meer of mindere mate nodig was en alle gezellige uurtjes koffie drinken. Matthew, jij stond altijd klaar als creatief brein op muzikaal gebied, als film deskundige en als expert voor artikelen in wetenschappelijke 'nonsens' bladen. Ik zal nooit vergeten dat je Olivia Newton-

John samen met me hebt gezongen. Remco, wij konden het van het begin af aan goed met elkaar vinden. Jij hebt me ondersteund met mijn werkzaamheden op het lab, tijdens het schaatsen, in de kroeg en indien nodig soms ook na sluitingstijd.

Mijn ouders zijn altijd in alles wat ik doe mijn steun en toeverlaat geweest. Mam, nu krijg ik eindelijk meer tijd om gezellig met je de stad in te gaan en een terrasje te pikken. Pap, met jouw kon ik altijd van gedachte wisselen als echte onderzoekers onder elkaar. Nu kunnen we weer eens ongestoord de natuur in trekken of een partijtje tennissen.

Tot slot wil ik Harry bedanken voor de goede zorgen tijdens het laatste gedeelte van het schrijven van mijn proefschrift. Ondanks dat je me graag veel vaker had willen zien, wilde je ook heel graag dat ik het proefschrift af zou ronden en heb je me gestimuleerd om door te gaan. Ik ben blij dat ik tijdens het lezen van artikelen in de trein toch kans heb gezien je te leren kennen!

Cindy.

Toen er voor het eerst een biosfeer ontstond, hebben zich onvermijdelijk veranderingen vertrokken in het scheikundige milieu van de aarde. Evenals de voedingsreserves in een kippeei, verschaften de rijkelijk aanwezige organische verbindingen waaruit het eerste leven zich heeft ontwikkeld het pasgeboren schepsel de nodige voedingsstoffen voor de eerste fase van zijn groei. In tegenstelling tot de situatie van het kuiken was er voor het leven echter slechts een beperkte voorraad voedingsstoffen voorhanden buiten het 'ei'. Zo gauw de vitale sleutelverbindingen eenmaal schaars werden zal het nog jonge levende wezen voor de keus zijn komen te staan om ofwel te verhongeren, ofwel te leren zelf zijn eigen bouwstoffen samen te stellen uit de meer elementaire grondstoffen van de omgeving, waarbij het zonlicht werd gebruikt als drijvende kracht. De noodzaak om dit soort keuzen te maken moet zich vele malen hebben voorgedaan en de verscheidenheid, onafhankelijkheid en weerbaarheid van de zich uitbreidende biosfeer hebben verhaast. Het kan ook gedurende deze tijd zijn geweest dat het idee van roofdier en prooi en van een voedselketen zich voor het eerst heeft ontwikkeld.

J.E. Lovelock, 1980. Gaia; de natuur als organisme. A.W. Bruna & Zoon, Utrecht, p. 35

Contents

Introduction

Defence mechanisms

Direct and indirect defence

In general, plants face severe circumstances under which they need to survive and reproduce. Under these strong selection conditions plants have evolved a defence against herbivory in a direct and/or an indirect way. Selection of plants in this evolutionary process takes place via plant fitness (Price et al., 1980; Vet, 1999). A direct way of a plant to defend itself from being eaten by herbivores is through its morphology such as glandular and non-glandular hairs, though outer layers and other barriers. Toxic, deterrent, antifeedant or antinutritional compounds can kill or deter the herbivorous insect, arrest their feeding or reduce the digestibility of the plant leaves. This contributes to the direct defence of the plant (Karban and Baldwin, 1997). However, toxicity of compounds is selective. Herbivores can adapt to or even use certain toxic compounds. An example of a specialized herbivore that is adapted to feed on the leaves of Solanaceae plants is the *Heliothis subflexa*. In contrast, *Heliothis virescens* is a generalist herbivore that feeds on a broad range of host plants (Sheck and Gould, 1996). Besides direct defence, plants have developed another way of defence through an interaction with carnivores (Dicke and Vet, 1999; Sabelis et al., 1999; Vet and Dicke, 1992), which is called indirect defence or extrinsic defence (Dicke, 1999; Price et al., 1980). This type of defence is characterized by the promotion of the effectiveness of natural enemies of the herbivores by offering alternative food or shelter (Dicke, 1999; Price, 1981; Price et al., 1980; Sabelis et al., 1999). In this way the plant can be helpful to predators or parasitoids that in turn can guard the plant from herbivores feeding on its leaves. The interaction that involves three levels of a foodweb is called a tritrophic interaction (Agelopoulos and Keller, 1994; Dicke, 2000; Du et al., 1996; Price, 1981; Vet and Dicke, 1992).

Induction of direct defence

Defence mechanisms are costly. As a consequence, some defence mechanisms are only induced at the moment that they are needed. For example, the production of proteinase inhibitors, oxidative enzymes and / or toxic constituents, can be newly induced or induced in larger amounts by the plant when the plant is attacked by herbivores or pathogens. Agrawal (1999) showed that induction of direct plant defenses led to an increase in plant fitness compared to non-damaged control plants. *Raphanus sativus* plants produced more seeds and were damaged less after early-season infestation by larvae of *Pieris rapae*. Moreover, Baldwin (1999) investigated the costs of jasmonate-induced responses and showed that the jasmonate mediated production of nicotine benefited tobacco plants that were subsequently attacked by herbivores. Induction of direct defence in a plant can also occur in response to volatile signals that reach the plant through its leaves. An example is methyl jasmonate released from sagebrush. This compound induced direct defence by increasing the production of polyphenol oxidase in neighbouring tobacco plants (Karban, 2001; Karban et al., 2000).

Induction of indirect defence

A sophisticated way of plant defence is the induction of volatiles after herbivore-infestation. This is also called induced indirect defence. The plants start to emit volatiles that differ from the volatiles of mechanically damaged leaves in a qualitative and / or quantitative way (Dicke, 1999; Dicke and Sabelis, 1988a). The herbivore-induced volatiles can attract predators or parasitoids that search for their prey or hosts. Moreover, the predators or parasitoids often show no or only a weak response towards volatiles emitted from leaves that are infested by herbivores that are not suitable as prey or hosts (Dicke, 1999; Dicke and Sabelis, 1988a; Dicke et al., 1998). Plants infested with different herbivore species emit volatile blends that differ in a qualitative or quantitative way. When the volatile blend shows qualitative differences, different signal transduction pathways are likely involved in the induction. Van Loon et al. (2000) reported that parasitoid

activity increases plant fitness in the system *Arabidopsis thaliana* - *Pieris rapae* caterpillars - *Cotesia rubecula* parasitoids. That attraction of parasitoids is beneficial to the plant has also been proven for the system *Zea mays* - *Spodoptera littoralis* larvae - *Cotesia marginiventris* or *Campoletis sonorensis* parasitoids (Fritzsche-Hoballah and Turlings, 2001). For predator-prey interactions no research on plant fitness has been done yet. Only has been investigated that leaf tissue removal in some plant species resulted in a higher seed production, while in other plant species this resulted in a lower seed production (Trumble et al., 1993).

Compatibility of direct and indirect defence

Plant species and plant cultivars can exploit direct and indirect defence in various ways. Plant genotypes that use both types of defence may have an advantage over other genotypes. However, a more sophisticated form of defence may also incur more costs to the plant. It is not known to what degree these two different defence mechanisms are compatible. An example of a competitive effect (antagonism) between direct and indirect defence has been documented for *Senecio jacobea* (Vrieling et al., 1991). Some genotypes have a high level of direct defence in the form of pyrrolizidine alkaloids, and consequently harbour fewer aphids on their leaves. Usually, ants visited the plants to collect the honeydew produced by these aphids. Besides, the ants also attacked caterpillars of the specialist herbivore *Tyria jacobeae* (indirect defence) that is not affected by the alkaloids in the leaves. Thus, a reduction in aphid and ant presence on plants with a strong direct defence leads to an increase in caterpillar damage because of a reduction in indirect defence. Also for *Nicotiana attenuata* a trade-off between direct and indirect defence has been reported (Kahl et al., 2000). When larvae of the nicotine-tolerant herbivore *Manduca sexta* feed on *Nicotiana attenuata* these plants increase the release of volatile terpenoids, but they do not increase the levels of nicotine, which is the plant's direct defence that can be exploited by *M. sexta*. An example that both direct and indirect defence can be

induced simultaneously in the plant is shown for tomato (Thaler et al., 2002). However, the induced indirect defence of tomato is not very specific, as only a few novel compounds, including methyl salicylate, are emitted compared to those emitted in response to mechanical damage (Dicke et al., 1998). These examples show that both defence mechanisms can be exploited by the plant.

Infochemicals

The herbivore-induced volatiles involved in a tritrophic interaction are called infochemicals. Infochemicals are chemicals that, in the natural context, convey information in an interaction between two individuals, evoking in the receiver a behavioural or physiological response that is adaptive to either one of the interactants or to both interactants (Dicke and Sabelis, 1988b). When infochemicals mediate an interspecific interaction and are beneficial to both emitter and receiver, they are called synomones (Dicke and Sabelis, 1988b) (Table 1).

Table 1: Terminology of infochemicals.

In intraspecific interactions:	
Pheromone:	mediates an interaction whereby the benefit is to the origin-related organism, to the receiver or to both
In interspecific interactions (allelochemicals):	
Allomone:	mediates an interaction whereby the benefit is to the emitter
Kairomone:	mediates an interaction whereby the benefit is to the receiver
Synomone:	mediates an interaction whereby the benefit is to both emitter and receiver

Isolation and identification of infochemicals is important for a better understanding of tritrophic interactions (Karban and Baldwin, 1997; Price, 1981, 1993; Sabelis et al., 1999). First, information about the behaviour of an insect towards infochemical blends must be gathered. Infochemicals can be present in

relatively large amounts that are dominant in the volatile blend, but they can also be present in minor amounts. Insects can be sensitive towards infochemicals that are present below the detection limit of the instrumental setup (Pickett, 1990). The chemical information can consist of one compound, but often two or more compounds are involved. In some synergistic interactions two or more compounds are present that show little or no activity when individually tested, but when tested together they are clearly bioactive. Synergistic effects are for example found in combinations of volatile plant compounds that can attract certain insect species (Fein et al., 1982; Ishikawa et al., 1984; Ladd, 1980; Visser, 1986; Weissbecker et al., 2000). Also synergism between plant odours and pheromones exists. In some cases the response of an insect can be increased when one or more plant compounds are added to a pheromone blend (Hedin et al., 1979; Wood, 1982). Different ratios between compounds in a pheromone blend are also important for insect attraction (Evans, 1984). Insects can distinguish between blends with such relative differences. They are stronger or sometimes even only attracted to the blend with component ratios that are related to their own species (Boo et al., 2000; Griepink, 1996). Moreover, they can distinguish between compounds with a different chirality (Duff et al., 2001; Zhang et al., 1997, 2000).

Tritrophic test system

Numerous different plant-herbivore-carnivore systems exist. One of these systems that has been investigated a lot is the system of plant-spider mite (*Tetranychus urticae*)-predatory mite (*Phytoseiulus persimilis*). *Tetranychus urticae* Koch is a polyphagous spider mite. It is a major pest on field crops, glasshouse crops, horticultural crops, ornamentals and fruit trees (Vrie et al., 1972). Moreover, the spider mite can easily adapt to plant varieties that have been selected for resistance, as was shown for varieties of tomato, broccoli and cucumber (Fry, 1989; Gould, 1979). This increases their threat as pest species even more. Dicke et al. (1990) showed that in response to the spider mite-

infestation, lima bean leaves started to produce novel volatiles compared to mechanically damaged leaves. To explain this, it was proposed that the spider mite's saliva contains elicitors that can induce a cascade of signal transduction steps in the plant. This was hypothesized because it has been shown that the regurgitant of *Pieris brassicae* and of *Spodoptera exigua* contains respectively the elicitors β-glucosidase and N-(17-hydroxylinolenoyl)-L-glutamine (volicitin) (Alborn et al., 1997; Mattiacci et al., 1995; Turlings et al., 1990). These elicitors can induce a volatile blend that is similar to the caterpillar-induced blend. *Tetranychus urticae*-infested plant leaves emit volatiles that can attract the specialist predatory mite *Phytoseiulus persimilis,* which exterminates spider mite populations (Sabelis and Dicke, 1985).

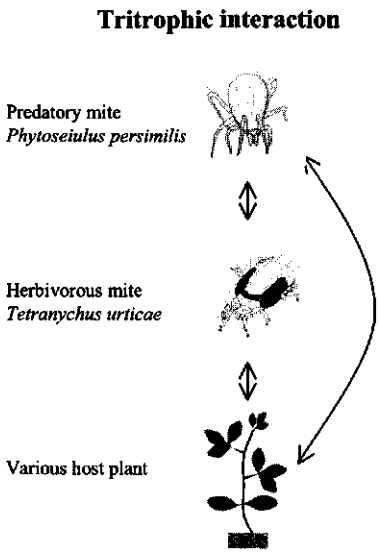

Figure 1: A tritrophic interaction between the herbivore *Tetranychus urticae*, the carnivore *Phytoseiulus persimilis* and various host plants. The arrows show that each organism can have an influence on organisms that are on a different level of the food web.

The predatory mite feeds especially on eggs but it also eats other spider mite stages. *Phytoseiulus persimilis* can travel long distances by dispersing through the air (Sabelis and Dicke, 1985) and it responds to odours that are released from plants that are infested by spider mites. The volatile profile emitted from spider mite-infested plants can differ qualitatively or quantitatively from the volatile profile emitted from mechanically damaged leaves (Dicke et al., 1998). This difference in the release of specific volatiles by the plant makes it worthwhile to investigate the degree of sophistication among the induction of volatile profiles from different plant species. In this thesis the tritrophic system of the spider mite *Tetranychus urticae*, the predatory mite *Phytoseiulus persimilis* and host plant species of the spider mite was investigated (Figure 1). *Tetranychus urticae* has a broad range of host plants and therefore, in this thesis eleven different plant species from different families are investigated.

Thesis outline

In this thesis a better insight is obtained in the degree of direct and indirect defence of the different test plant species against the spider mite *T. urticae*. It was hypothesized that when a plant possesses a strong direct defence it will not invest in a strong indirect defence and vice versa. To investigate this, different experiments were carried out with various plant species to compare their direct and indirect defence levels. Additionally, a more efficient and reliable fractionation method was developed for biological identification of compounds that show bioactivity.

The acceptance by the spider mite of several plant species was investigated as a measure of the plant's direct defence, which is discussed in Chapter 2. Indirect defence, i.e. the attraction of predatory mites towards plant volatiles that are released by spider mite-infested plant species was investigated for a number of plant species that were already investigated with respect to the level of direct defence (Chapter 3). It was hypothesised that plant species with a weak direct

defence would invest in the production of novel compounds. This specificity in odour production of several plant species and the specificity of the spider mite-induced compounds can lead to a more reliable odour source for the predatory mites. Therefore, the production of novel compounds was analysed in the volatile blend of spider mite-infested leaves of plant species and compared to clean or mechanically damaged leaves (Chapter 4). The plant species that were used had already been investigated with regard to their direct and indirect defence (Chapter 2 and 3).

Besides information on the defence of separate plant species, also data on defence at the level of plant families were compared. Therefore, two plant families were chosen that were expected to differ in their degree of direct defence. Plant species of the Fabaceae are often susceptible to a larger number of herbivores than plants of the Solanaceae. Plants of the latter family have more specialist herbivores feeding on their leaves. These herbivores are adapted to the plant's toxins, such as alkoloids (Campo and Renwick, 2000; Mullin et al., 1997; Sheck and Gould, 1996). The results obtained from different plant species that are members of these families were compared (see Chapter 2, 3 and 4).

Infochemicals are often part of a complex volatile mixture. Conventional bioassays that are used to show the biological activity of a compound have shown some disadvantages. The ratios of compounds in the blend can change due to trapping and desorption techniques and, besides, solvent introduction into a bioassay can disturb the insect's response. Insects can distinguish between these relative differences (Boo et al., 2000; Griepink, 1996). Therefore, a system to fractionate odour mixtures was developed (Chapter 5), in which compounds can selectively be removed from the mixture. With this method the attraction of predatory mites was tested towards fractions of a volatile plant mixture with compounds that are present in the same ratios as in the intact odour blend.

Besides, the method was validated by calculation of the recoveries of reference compounds.

In Chapter 6 the degree of direct and indirect defence for different plant species and plant families is discussed. Additionally, the hypothesis that the degree of direct defence of a plant will affect its degree of indirect defence and vice versa will be discussed. Furthermore, the effectiveness of the newly developed method that can be used to isolate and identify volatile infochemicals from a complex blend will be described and evaluated. Besides, future perspectives will be discussed.

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Differences among plant species in acceptance by the spider mite *Tetranychus urticae* Koch

Abstract

The spider mite *Tetranychus urticae* Koch has a broad range of host plants. However, the spider mite does not accept all plants to the same degree due to differences in nutritive and toxic constituents. Other factors, such as the induction of secondary metabolites, the morphology of a leaf surface and the presence of natural enemies also play an important role in plant acceptance. We compared plants from various families in their degree of acceptance by the spider mite, to get an indication of the plant's direct defence. *Glycine max* (soybean), *Humulus lupulus* (hop), *Laburnum anagyroides* (golden chain) and *Nicotiana tabacum* (tobacco) were highly accepted by the spider mites. Different glandular hair densities among tobacco cultivars did not affect their suitability towards spider mites significantly. *Solanum melalonga* (eggplant), *Robinia pseudo-acacia* (black locust), *Vigna unguiculata* (cowpea) and *Datura stramonium* (thorn apple) were accepted by the spider mites to a lesser degree. *Vitis vinifera* (grapevine) was poorly accepted by the spider mite. It might be that the food quality of the leaves was not high enough to arrest the spider mites. Also *Capsicum annuum* (sweet pepper) and especially *Ginkgo biloba* (ginkgo) were poorly accepted by the spider mite, probably due to the presence and concentration of certain secondary metabolites in the leaves. The spider mites accepted all plants belonging to the Fabaceae for feeding, but those belonging to the Solanaceae showed a large variation in spider mite acceptance varying from well accepted (tobacco) to poorly accepted (sweet pepper).

This Chapter was accepted by the Journal of Applied Entomology

Introduction

Tetranychus urticae Koch is a polyphagous, parenchyma cell feeding spider mite with over 200 host plant species. It is a major pest on field crops, glasshouse crops, horticultural crops, ornamentals and fruit trees (van de Vrie et al., 1972). Moreover, it can easily adapt to plant varieties that have been especially selected for resistance, as was shown for e.g. tomato, broccoli and cucumber (Fry, 1989; Gould, 1979). This increases their threat as pest species even more. Their host plants differ in food quality, which does not only depend on the level of primary plant metabolites, but also on the quantity and nature of secondary metabolites. These secondary metabolites can function as toxins, deterrents and digestibility reducers (Rosenthal and Berenbaum, 1991). Deterrent and antifeedant effects on spider mites were, for example, shown for ginkgo and tobacco (Dabrowski, 1973).

Another variation factor in food quality is the plant's response to herbivory with local and/or systemic induction of secondary metabolite synthesis (Karban and Myers, 1989; Karban and Baldwin, 1997; Tollrian and Harvell, 1999). These induced responses can be herbivore specific (Stout et al., 1994) and can lead to reduced mite fecundity in e.g. soybean (Brown et al., 1991) or cotton (Karban and Carey, 1984). Hildebrand et al. (1986, 1989) showed that spider mite damage on soybean increased the lipid peroxidation level and the lipoxygenase activity, which are correlated to the level of resistance in soybean.

Furthermore, the morphology of the leaf surface, such as a thick cuticle or glandular and non-glandular hairs, can have a defence function by reducing herbivory. Glandular hairs can have an effect on the spider mite population by entrapment of the spider mites and subsequent dehydration (Patterson et al., 1974). An example is the negative effect of glandular hairs on the dispersal behaviour of both *T. urticae* and its natural enemy *Phytoseiulus persimilis* on tomato plants (van Haren et al., 1987).

Besides the chemical value of a plant leaf and the structure of its surface, risks from natural enemies can be of great importance to a herbivore. Some plants offer shelter or alternative food to predators and thus create an enemy dense environment, while other plants constitute a relatively more enemy free space (Dicke, 2000). To avoid an enemy dense leaf surface, herbivores may prefer an inferior food source that is free of enemies, as has been shown for *Pieris napi* by specialising on *Arabis* plants (Ohsaki and Sato, 1994). Moreover, Strong et al. (1997) showed that *T. urticae* has special refugia on hop where its predator *Neoseiulus fallacis* does not occur.

Host selection by herbivores is therefore expected to be affected by the plant's nutritional value, the plant's physical characteristics and the chances of encountering carnivorous enemies on a plant (Schoonhoven et al., 1998; Bernays and Chapman, 1994). Furthermore, spider mites are known to differentially accept different plant species (Yano et al., 1998). We have investigated plants that were expected to differ in types and quantities of secondary metabolites, to compare their degree of acceptance by the spider mite, as an indication of the plant's direct defence. The plant species were selected on the basis of their economical relevance and their expected level of defence against spider mites. Soybean, hop and grapevine were selected because spider mites are a serious pest on these plant species. This indicates that they are good host plants for the spider mites, although some differences in susceptibility exist (Wheatley and Boethel, 1992; Leszczynski et al., 1988; Peters and Berry, 1980b; Schruft 1985). *Tetranychus urticae* is the most important spider mite pest of grapevines in European regions with a dry summer (Schruft 1985). On the other hand, we also investigated plants that were known for their strongly deterrent and antifeedant secondary metabolites, such as alkylphenols and terpene trilactones in ginkgo and nicotine in tobacco (Dabrowski, 1973; Matolscy, 1988; Fu-shun et al., 1990).

Besides the investigation of species belonging to various families, two plant families, the Fabaceae and the Solanaceae, were examined in closer detail. The Solanaceae comprise medicinal plants, food plants and many poisonous plants (Hegnauer and Hegnauer, 1973). Some herbivore species have adapted to these toxic plant components and became specialists. For example, the specialist herbivore Colorado potato beetle (*Leptinotarsa decemlineata*) specializes on Solanaceae species that are rich in alkaloids. It proved to be less sensitive to the antifeedant effects of alkaloids than the generalist herbivore western corn rootworm, *Diabrotica virgifera* (Mullin et al., 1997). Besides, literature has shown that *Manduca sexta* is a specialist on Solanaceous plants (del Campo and Renwick, 2000; Mechaber and Hildebrand, 2000). The Fabaceae also comprise various food plants, but in contrast to the Solanaceae they have more generalist herbivores feeding on the plant leaves. The moth species *Heliothis virescens* is such a generalist herbivore, feeding on plants from at least fifteen plant families, while another related species, *Heliothis subflexa*, feeds only on plants of the Solanaceae (Sheck and Gould, 1996). Therefore, plants of the Solanaceae were expected to have a higher degree of direct defence and to be less accepted by the generalist spider mite *T. urticae* than plants of the Fabaceae.

Materials and methods

Plant material.

The investigated test plants, their family and cultivar name, age and mean leaf area are shown in Table 1. The test plants were selected from five different families, with 4 plant species taken from both the Fabaceae and the Solanaceae. The age of each plant was determined from the day of seeding until the week of the first experiment. The mean leaf area was calculated as the average of 10 leaf areas, unless mentioned otherwise. The leaves were measured with an area meter (Li-Cor LI-3100). All plants were grown at the greenhouse facility of Wageningen University, except for the ginkgo trees (0.5 m), the black locust trees (1-1.5 m), the grapevines (0.5-1 m) and the golden chain (fully expanded tree of about 15 m), which all grew outdoors in Wageningen. The greenhouse had a temperature of 20 ± 2 °C, a relative humidity of 60-80 %, and a photoperiod of L16:D8 hours. In the experiments we only used fully expanded leaves, and in the case of soybean, cowpea and the control plant lima bean these were fully expanded trifoliar leaves.

Spider mites.

Two-spotted spider mites, *Tetranychus urticae* Koch (Acari: Tetranychidae), were reared on lima bean plants. These plants were grown in a greenhouse at a temperature of 20-30 °C, a relative humidity of 60-80 % and a photoperiod of L16:D8. Well-fed adult female spider mites were used in the experiments.

Table 1: Names of plants and trees used.

Family	Genus	Species	Common name	Cultivar	Age (weeks)	Mean leaf area ± SD (cm^2)
Fabaceae	*Glycine*	*max*	soybean	Gieso	5	115 ± 10
Fabaceae	*Laburnum*	*anagyroides*	golden chain		- [1]	20 ± 5 [3]
Fabaceae	*Phaseolus*	*lunatus*	lima bean		4	185 ± 40
Fabaceae	*Robinia*	*pseudo-acacia*	black locust		- [1]	15 ± 5 [3]
Fabaceae	*Vigna*	*unguiculata*	cowpea	Black eye	4	160 ± 25
Ginkgoaceae	*Ginkgo*	*biloba*	ginkgo		- [1]	15 ± 5
Moraceae	*Humulus*	*lupulus*	hop		16 [2]	20 ± 5 [3]
Solanaceae	*Capsicum*	*annuum*	sweet pepper	Lambada	12	120 ± 20
Solanaceae	*Datura*	*stramonium*	thorn apple		6	115 ± 25
Solanaceae	*Nicotiana*	*tabacum*	tobacco	SR1	8	125 ± 35
Solanaceae	*Nicotiana*	*tabacum*	tobacco	I-35	11	40 ± 5
Solanaceae	*Nicotiana*	*tabacum*	tobacco	Speight 33	11	50 ± 10
Solanaceae	*Solanum*	*melalonga*	eggplant	Black beauty	8	260 ± 25
Vitaceae	*Vitis*	*vinifera*	grapevine	Glorie van Boskoop	- [1]	165 ± 20

[1] Age not known, but ≥ 3 years

[2] Age calculated from the time the plant has been vegetatively propagated

[3] An average of 50 leaves was taken to calculate the average leaf size

Host plant acceptance.

Two square leaf sections of 1 cm^2 were placed on top of a moist filter paper in a Petri dish. The leaf sections were excised near the main vein from a fresh leaf and placed ventral side up. A leaf section of a test plant was placed on one side of the Petri dish and a leaf section of lima bean was placed on the other side as a control (trap). The leaf sections were connected via a plastic bridge (length: 5 cm, width: 1 cm, thickness: 1 mm, Figure 1A). At the beginning of every experiment 10 spider mites were placed on the test plant leaf section in all Petri dishes, alternately on the left or right side of the Petri dish.

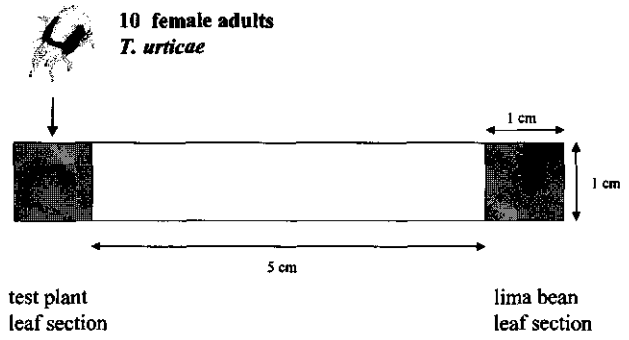

Figure 1A: To investigate plant acceptance, 10 spider mites (*T. urticae*) were placed on the test leaf disc. On the other side of the Petri dish a leaf disc of lima bean was placed, which functioned as a trap for spider mites that migrated from the test plant section.

If the test plant was not a good host plant for the spider mites, the mites started wandering around in search of another food source and crossed the bridge. There, they encountered the lima bean leaf section where they could start to feed, because lima bean is an excellent host plant for spider mites. After all mites had been placed on the test leaves, we observed the distribution of the spider mites over the test and control leaf in each Petri dish after 15 minutes, 1, 2 and 3 hours to assess the migration rate of the spider mites over time. The experiments were carried out on 4 different days with 6 Petri dishes per day, which means that 24 replicate choice experiments were made with a total number of 240 spider mites per host plant. Drowned mites (less than 4 % of the total number of spider mites for each plant) were excluded from the distribution calculations. To avoid a water film between the leaf disc and the bridge, leaf sections without veins or with just a few small veins were selected because these veins could lift the bridge.

Because the leaf sections of ginkgo were very curly they were fixed onto wet cotton wool with small pins.

Influence of glandular hair densities on spider mites.

The influence of the glandular hair density of the tobacco cultivar SR1 was tested, because the stickiness of their hairs could influence the spider mites' ability to move. We selected two closely related tobacco cultivars, I-35 and Speight 33, and used the same set-up as was used to test the host plant acceptance by the spider mites (Figure 1A). The cultivar I-35 has a low density of glandular hairs and the cultivar Speight 33 has a high density of glandular hairs. With the exception of tobacco, none of the test plants that were used in our experiments seemed to hinder the spider mites' ability to move.

Attraction to plant odours.

To investigate whether the spider mites' behaviour in the setup of Fig. 1A was affected by attraction to odour from the lima bean leaf section, a two-choice experiment was carried out. For this, a T-shaped bridge (length long side: 5 cm, length short side: 3 cm, width: 1 cm, thickness: 1 mm) was connected to a lima bean leaf section and a plastic section (Figure 1B). A spider mite was individually placed at the bottom of the bridge and allowed to walk to the T-junction, where it had to make a choice. Once the mite entered one of the sections, its choice was recorded. Per day 20 mites were observed. A new lima bean odour source was taken for every ten mites, and alternately placed at the left or right side of the bridge. In total the test was replicated on five different days.

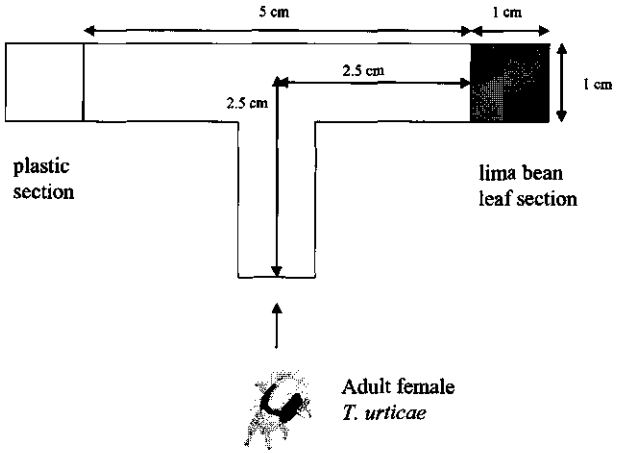

Figure 1B: A two-choice setup to investigate odour attraction of spider mites to the lima bean leaf section. A spider mite was placed on a T-shaped bridge and had to make a choice at the T-junction between a lima bean leaf section and a plastic section. In total 100 spider mites were allowed to make a choice.

Statistics.

For each spider mite distribution on a different host plant, a 95% confidence interval was calculated using a student's t-distribution (n = 24 Petri dishes). The spider mite distribution on different host plants was classified in groups with overlapping confidence intervals. Thus, a significant difference in spider mite distribution between the groups is shown. To test whether the spider mites were attracted to the odours of lima bean, the total number of mites arriving at each disc was counted and subjected to a χ^2-test (n = 100 spider mites). To analyse whether the distribution of spider mites on the tobacco cultivars with a high and low density of glandular hairs was significantly different a 2*2 contingency table was used. For this test each Petri dish was categorised on having more spider mites on the test plant or on the control.

Results

Host plant acceptance.

The mean distribution of the spider mites over the test plant leaf and lima bean leaf over the 24 Petri dishes is shown in Figure 2, three hours after the introduction of the spider mites.

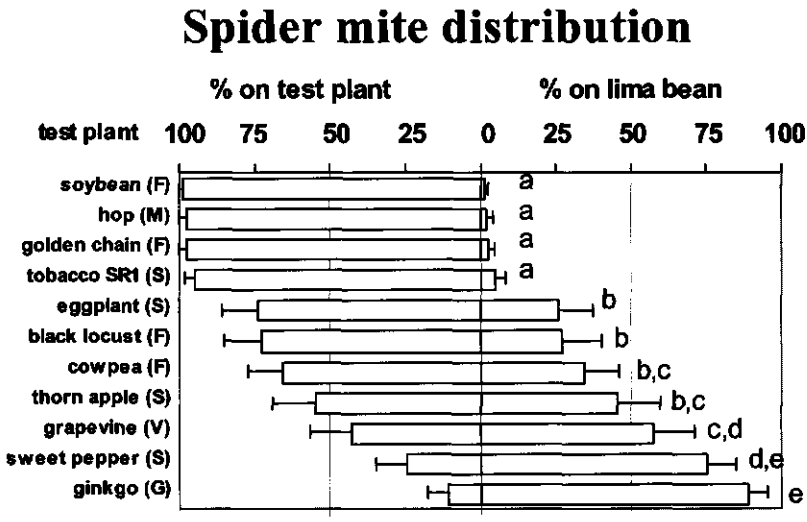

Figure 2: Plant acceptance by the spider mite *T. urticae*, 3 hours after the spider mites had been placed on the test leaf discs. The distribution of the spider mites over the test and the control leaf disc was calculated (n = 24 Petri dishes) and the mean distribution values and the 95% confidence intervals are shown. The spider mite distribution was classified in groups with overlapping confidence intervals. Significant differences are indicated with different letters. The different plant families depicted in the figure are: F = Fabaceae, G = Ginkgoaceae, M = Moraceae, S = Solanaceae and V = Vitaceae.

With soybean (99 ± 1 % (mean ± 95 % confidence interval)), hop (98 ± 1 %), golden chain (98 ± 1 %) and tobacco SR1 (95 ± 2 %) as test plant, more than 90 % of the spider mites had settled on the test plant leaf after three hours. Eggplant,

black locust, cowpea and thorn apple had a lower percentage of spider mite acceptance (74 ± 6 %, 73 ± 6 %, 65 ± 6 % and 55 ± 7 % respectively). Grapevine (43 ± 7 %), sweet pepper (25 ± 5 %) and ginkgo (11 ± 3 %), showed the lowest acceptance of the test plant by the spider mites after three hours.

Migration rate.

The percentage of the spider mites on different host plants is plotted as a function of time in Figure 3. Soybean, hop, golden chain and tobacco had a low spider mite migration rate. When these plant species were tested, almost all the spider mites stayed on the test leaf during the whole period of three hours. Eggplant, black locust, cowpea, thorn apple and ginkgo had a high initial migration rate of the spider mites to the lima bean, but this migration stabilised after two hours when most of the spider mites had already made a choice. Grapevine and sweet pepper also had a high initial spider mite migration rate, but the percentages of spider mites on the test plant were still decreasing after two hours.

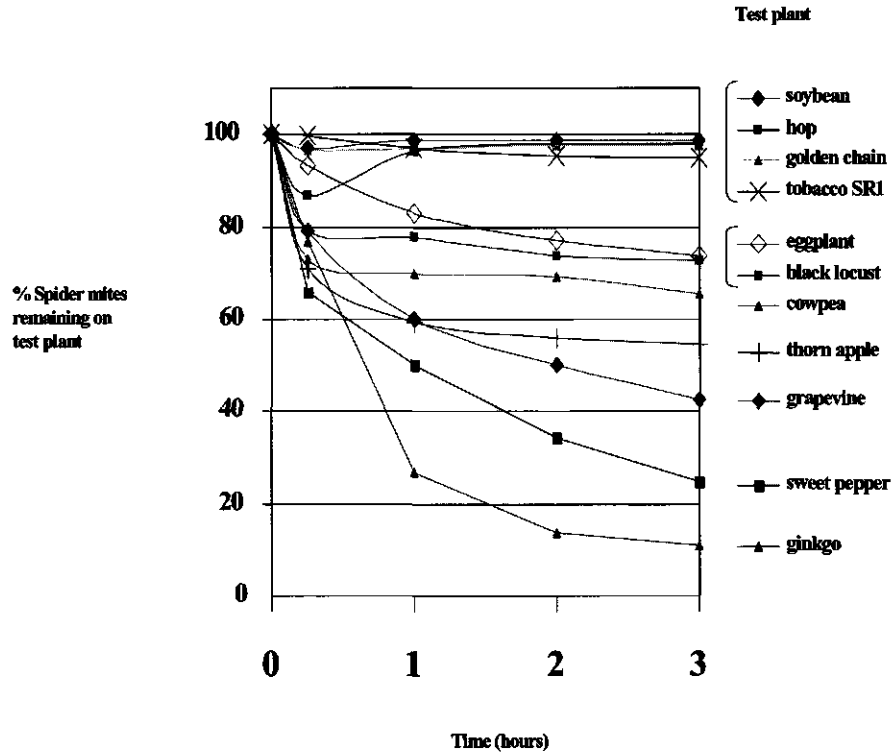

Figure 3: Spider mite acceptance of different host plants over time. The percentage of spider mites remaining on the test plant (n = 240) is depicted.

Comparison of tobacco cultivars with different densities of glandular hairs.

The comparison of the tobacco cultivars with high and low densities of glandular hairs is shown in Figure 4. The distribution of the spider mites on both cultivars was in favour of the tobacco leaf sections, 91 % on I-35 and 81 % on Speight 33. The difference between the spider mites' distribution on both cultivars was not significant (2*2 contingency table, P = 0.47 with Yates correction).

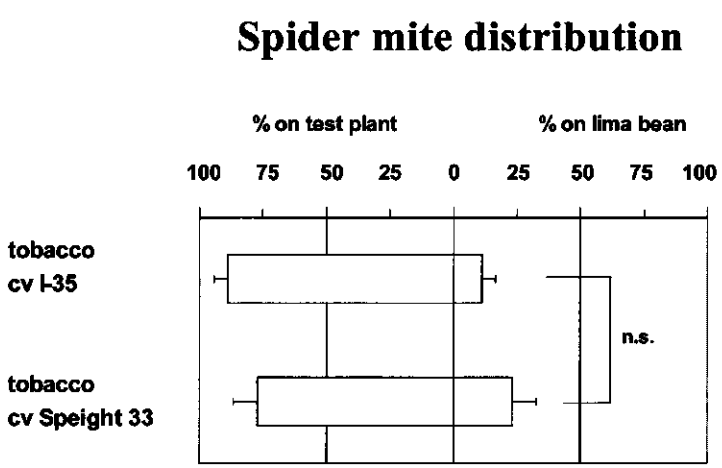

Figure 4: The differences in plant acceptance by spider mites between two closely related tobacco cultivars are depicted, I-35 (low density of glandular hairs) and Speight 33 (high density of glandular hairs). The mean distribution values and the 95% confidence intervals were calculated (n = 24 Petri dishes). The Petri dishes were categorised on having more spider mites on the test plant or the control. A 2*2 contingency table was used to investigate if a significant difference was present.

*** = $P \leq 0.001$, ** = $0.01 \geq P > 0.001$, * = $0.05 \geq P > 0.01$, n.s. = $P > 0.05$

Attraction to plant odours.

We tested the influence of odours from the lima bean leaf section. In total 44 out of 100 spider mites tested walked to the lima bean leaf section. Thus, no significant attraction ($P > 0.05$) of the spider mites to plant odour was found at a distance of 2.5 cm from the T-junction to the leaf section.

Discussion

Plant acceptance: comparison of plant species.

From the host plant acceptance experiments we can conclude that the plant species vary in their degree of acceptance by the *T. urticae* population. Soybean, hop, golden chain and tobacco are highly acceptable to the spider mite, because almost one hundred percent of the spider mites stayed on the plant. Tobacco, however, was a highly accepted host plant as well, despite of the nicotine and other alkaloids in its leaves which are known to be toxic to spider mites in certain concentrations (Matolcsy et al., 1988). Eggplant, black locust, cowpea and thorn apple were also accepted by the spider mite, but some migration to the lima bean leaf took place. In the case of grapevine and sweet pepper, the majority of the spider mites migrated. Grapevine leaves contain a lot of flavonoids, tannins and volatile terpenes. An outbreak of spider mites on the grapevines in our greenhouse (personal observation) suggests that it is likely that these components are not deterring the spider mites. An explanation for this might be that the food quality of the leaves used in the experiments was not high enough to arrest the spider mites. For sweet pepper and most of the other investigated plants there are no phytochemical reports on their leaf contents known to us. Ginkgo was least accepted by the spider mite. Almost all the mites migrated to the lima bean leaf disc. This result was expected, because the ginkgo tree has long been known for its effective antifeedants (Major, 1967). Their leaves contain alkylphenols and terpene trilactones, which are known to be toxic to spider mites (Dabrowski, 1973) and are effective deterrents for caterpillars (Matsumoto and Sei, 1987; Fu-shun et al., 1990).

Plant acceptance on family level.

At the level of plant families we see a difference in spider mite acceptance between the Fabaceae and Solanaceae. The plants of the Fabaceae were all accepted for feeding, with soybean and golden chain as highly acceptable host

plants for the spider mites. The plants of the Solanaceae showed a larger variation in acceptance. On the one hand tobacco was highly accepted by the spider mites, but on the other hand sweet pepper had a low degree of acceptance. We hypothesised that plants of the Solanaceae were better directly defended than plants of the Fabaceae. However, this hypothesis is not supported by our data for the spider mite *Tetranychus urticae* because some species from the Solanaceae, such as tobacco and eggplant, were highly acceptable to the spider mites. The mechanism that explains the acceptance of the host plants investigated remains to be investigated.

Comparison of tobacco cultivars with different densities of glandular hairs.
The differences in densities of glandular hairs on tobacco did not significantly affect the degree of acceptance by the spider mites. However, in other experiments we noticed a much lower survival rate when spider mites were reared on the tobacco cultivar Speight, with a high glandular hair density, than on cultivar I-35, with a low glandular hair density (unpublished data). This means that the morphology can play a role in the direct defence. Sticky glandular hairs hinder the mobility of the spider mite and the spider mite larvae may have been poisoned by toxic components or entrapped by the sticky exudate of the glandular hairs, as shown for several tobacco varieties by Patterson et al. (1974). On strawberry (Luczynski et al., 1990) and tomato (Rodriguez et al., 1972) mite survival and oviposition were also negatively correlated with the density of (non)-glandular hairs. However, an increase in oviposition of *T. urticae* was shown for hop cultivars with a higher density of ventral and glandular hairs (Peters and Berry, 1980a).

Attraction to plant odour.
The odour test shows that *T. urticae* was not influenced by the odour of lima bean in the current setup, although the spider mite uses lima bean odour to find its host plant as was shown with a much larger plant biomass of 7 lima bean

leaves in an olfactometer test (Dicke, 1986). The size of the leaf section used in the host plant acceptance experiments was only 1 by 1 cm^2 and most probably too small to have an influence on the mites' behaviour because we did not find a significant attraction in the experiment with the T-bridge. Therefore, our results are an indicator of plant acceptance and not attraction to plant species.

Plant acceptance in relation to spider mite reproduction.

Although plant acceptance gives an indication of direct plant defence, the reproduction rate of spider mites on a host plant is important as well. Yano et al. (1998) showed a positive correlation between the host plant acceptance of spider mites after one day and the mean number of eggs produced by these mites in 5 days. Besides some wild herbaceous plants, they investigated the following cultivated plants, *Fragaria* sp. (strawberry) from the Rosaceae, *Chrysanthemum* sp. from the Asteraceae, *Phaseolus lunatus* and *P. vulgaris*, both from the Fabaceae. Especially the spider mites on plants from the Fabaceae (*P. lunatus* and *P. vulgaris*) showed a high plant acceptance and a high fecundity. However, on plants where more than 50 % of the spider mites migrated to the kidney bean leaf (*P. vulgaris*) after one day, the fecundity was very low or zero. This correlation suggests that spider mites could easily reproduce on many of our test plants, especially on soybean, hop, golden chain and tobacco. Peters and Berry (1980b) showed that on hop varieties that differed in susceptibility the spider mites had similar oviposition rates, but the development times increased on more resistant cultivars. In the case of grapevine, sweet pepper and ginkgo, which showed a spider mite migration of more than 50 % in our experiments, the fecundity might be negatively affected. The ginkgo tree is especially known for its strong repellent effect on herbivores. The fecundity of the spider mites is expected to be almost zero, because they do not survive the intake of toxic ginkgo leaf constituents (Dabrowski, 1973). Moreover, we did not succeed in rearing spider mites on ginkgo (personal observation), while the rearing of spider mites on other plants was successful.

Conclusion.

The investigated test plants varied strongly in their degree of spider mite acceptance. Most plant species were suitable as a host plant for the spider mite. Ginkgo, sweet pepper and grapevine were the least preferred plants for *T. urticae*. We hypothesised that plants of the Solanaceae were better directly defended than plants of the Fabaceae. However, this hypothesis is not supported by our experiments, because the spider mite *Tetranychus urticae* did accept some plant species from the Solanaceae, such as tobacco and eggplant. We investigated plant acceptance by the spider mite, as an indication for the degree of a plant's direct defence. A positive correlation between plant acceptance by the spider mite and their fecundity was revealed by Yano et al. (1998). We currently investigate the degree in which different plant species release spider mite induced volatiles that attract predatory mites, as a measure of indirect defence. In doing so, we will compare the relative strength of direct and indirect plant defence for the plants described in this paper.

Acknowledgements

We would like to thank Herman Dijkman for the supply of spider mites. Henk Smid took care of the plants in the greenhouse. The tobacco seeds were supplied by Verne A. Sisson (N.C. State University, Oxford Tobacco Research Station, Oxford, USA). Gerrit Gort gave us valuable advice on the statistics. Furthermore we thank the PhD-group of the laboratory of Entomology for their comments on this manuscript.

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Attraction of *Phytoseiulus persimilis* (Acari: Phytoseiidae) towards volatiles from various *Tetranychus urticae*-infested plant species

Abstract

Plants infested with the spider mite *Tetranychus urticae* Koch, may indirectly defend themselves by releasing volatiles that attract the predatory mite *Phytoseiulus persimilis* Athias-Henriot. Several plants from different plant families that varied in the level of spider mite acceptance were tested in an olfactometer. The predatory mites were significantly attracted to the spider mite-infested leaves of all test plant species. No differences in attractiveness of the infested plant leaves were found for predatory mites reared on spider mites on the different test plants or on lima bean. Thus, experience with the spider mite-induced plant volatiles did not affect the predatory mites. Jasmonic acid was applied to ginkgo leaves to induce a mimic of a spider mite-induced volatile blend, because the spider mites did not survive when incubated on ginkgo. The volatile blend induced in ginkgo by jasmonic acid was slightly attractive to predatory mites. Plants with a strong direct defence were thought to invest less in indirect defence than plants with a weak direct defence. However, plants that had a strong direct defence such as ginkgo and sweet pepper, did emit induced volatiles that attracted the predatory mite. This indicates that a combination of direct and indirect defence is to some extent compatible in plant species.

This Chapter was published in Bulletin of Entomological Research 96, 2002, p. 539-546.

Introduction

Plant species show variation in the direct defence mechanisms that protect them against insect herbivory, such as the composition of toxic secondary metabolites, proteinase inhibitors, spines and glandular hairs (Rhoades & Cates, 1976; Rhoades, 1985; Rosenthal & Berenbaum, 1991). Besides direct defence, plants can also defend themselves indirectly. This is done by promoting the effectiveness of natural enemies of the herbivores, for example by offering alternative food or shelter (Price *et al.*, 1980; Price, 1981; Sabelis *et al.*, 1999; Dicke, 1999a). Another way of indirect defence is to attract predators by the release of herbivore-induced volatiles, which differ from the volatiles of mechanically damaged leaves in a qualitative and / or quantitative way (Dicke & Sabelis, 1988a; Dicke, 1999a). The volatile infochemicals induced in plants by herbivory that play a role in the attraction of the predators are called synomones (Dicke & Sabelis, 1988b).

The use of these two types of defence, direct and indirect defence, may vary between plant species and plant genotypes. From an evolutionary point of view, plant genotypes that use both types of defence may have an advantage over other plant cultivars, but they may also incur more costs. It is not known to what degree these two different defence mechanisms are compatible. A trade-off between direct and indirect defence has been documented for *Senecio jacobaea* Linnaeus (Asteraceae) (Vrieling *et al.*, 1991). On genotypes with increased levels of pyrrolizidine alkaloids (direct defence) fewer aphids of *Aphis jacobaeae* Schrank (Homoptera: Aphididae) were found. As a consequence, fewer ants visited these genotypes to collect aphid-produced honeydew. The ants also attack caterpillars of the specialist herbivore *Tyria jacobaeae* L. (Lepidoptera: Arctiidae) that is not affected by the alkaloids (indirect defence). Thus, a reduction in aphid and ant presence on plants with a strong direct defence leads to an increase in caterpillar damage because of a reduction in indirect defence.

Also for *Nicotiana attenuata* Torr. Ex. Watson (Solanaceae) a trade-off between direct and indirect defence has been reported (Kahl *et al.*, 2000). When *Manduca sexta* (Johannsen) (Lepidoptera: Sphingidae) larvae feed on *N. attenuata* these plants increase the release of volatile terpenoids, but they do not increase the levels of nicotine, which is the plant's direct defence.

The spider mite *Tetranychus urticae* Koch (Acari: Tetranychidae) is a generalist herbivore and a serious pest in many crops. One of its natural enemies is the predatory mite *Phytoseiulus persimilis* Athias-Henriot (Acari: Phytoseiidae), which specializes on spider mites in the genus *Tetranychus* and exterminates spider mite populations (Sabelis, 1981). Behavioural evidence that the predatory mite *P. persimilis* is attracted to *T. urticae*-induced plant volatiles has been presented for lima bean (Fabaceae), tomato (Solanaceae), cucumber (Cucurbitaceae), gerbera (Asteraceae), ground ivy (Lamiaceae), cotton (Malvaceae) and for rose, pear and apple (Rosaceae) (Sabelis & van de Baan, 1983; Dicke & Sabelis, 1988a; Dicke *et al.*, 1990a; Dicke *et al.*, 1990b; Bruin *et al.*, 1992; Takabayashi *et al.*, 1994a; Krips *et al.*, 1996). Moreover, it was shown that the predatory mite *P. persimilis* is not attracted to odours from the spider mite *T. urticae* (Sabelis & van de Baan, 1983; Sabelis *et al.*, 1994). This means that the attraction of the predatory mite *P. persimilis* to *T. urticae*-infested plants cannot be explained by herbivore-derived volatiles but is caused by plant-derived volatiles. This has been supported by additional studies. Chemical analysis has resulted in the identification of the spider mite-induced components in the volatile blend of infested lima bean when compared to mechanically damaged leaves (Dicke *et al.*, 1990a). Also for apple, cucumber, tomato and gerbera plants differences in odour profiles were found between volatiles emitted in response to spider mite-damage and to mechanical damage (Dicke *et al.*, 1998; Krips *et al.*, 1999; Takabayashi *et al.*, 1991; Takabayashi *et al.*, 1994a). Dicke et al. (1990a) showed that individual compounds found in the odour blend of spider mite-

infested leaves attracted *P. persimilis*. Besides local induction also systemic induction of non-infested leaves of the same plant species was found to take place after one leaf of a lima bean plant was infested by *T. urticae* (Dicke *et al.*, 1990b; Dicke, 1994). Finally, treatment of Lima bean or gerbera plants with the plant hormone jasmonic acid resulted in (a) the emission of a volatile blend that is qualitatively similar to that induced by spider mite feeding and (b) the attraction of the predatory mite *P. persimilis* (Dicke et al. 1999, Gols et al. 1999).

There is variation in herbivore-induced volatile production between different plant species and cultivars (Takabayashi *et al.*, 1994a; Takabayashi & Dicke, 1996). Some spider mite-infested plant species emit new components that are not emitted when these plants are mechanically damaged. Other spider mite-infested plant species do not emit new components, but their volatile blends differ in a quantitative way from the blend emitted from mechanically damaged plants. Also, different spider mite species feeding on the same plant species induce qualitatively or quantitatively different odour blends and the predatory mite *P. persimilis* can distinguish between these different induced blends (Sabelis & van de Baan, 1983; Sabelis & Dicke, 1985; Dicke & Sabelis, 1988a; Takabayashi *et al.*, 1991). Moreover, the behavioural response of predatory mites can be affected by experience (Dicke *et al.*, 1990b; Krips *et al.*, 1999; Drukker *et al.*, 2000). *Phytoseiulus persimilis* does not respond to *T. urticae* infested cucumber or gerbera leaves when the predatory mites were reared on *T. urticae* on lima bean. However, after experience with *T. urticae* feeding on cucumber or gerbera, the predator is attracted by the volatiles from these infested plants (Dicke *et al.*, 1990b; Krips *et al.*, 1999). Drukker *et al.* (2000) showed that naïve predatory mites responded positively to odours from an unfamiliar environment, after these mites had had a positive experience with prey in combination with the unfamiliar odour.

In this paper the attraction of *P. persimilis* to *T. urticae*-induced plant volatiles of several plant species from different families is investigated. Furthermore, the effect of two different rearing histories is investigated, to assess whether experience with the volatiles from the spider-mite-infested test plant has an effect on the predatory mite's response. Plant species have been selected that differ in their degree of direct defence (van den Boom *et al.*, 2003). However, indirect defence against spider mites has not been investigated for these plant species yet, although for some of them indirect defence against other herbivore species has been documented. We would like to know to what degree a plant defends itself and to what extent direct and indirect defence mechanisms are interrelated. Special emphasis is placed on plants from the Fabaceae and the Solanaceae. Plant species from the Fabaceae seem to have a less efficient direct defence compared to plant species from the Solanaceae (van den Boom *et al.*, 2003). To defend themselves against herbivory, plants of the Fabaceae are therefore expected to have a relatively stronger indirect defence than plant species from the Solanaceae.

Materials and methods

Plant material

The test plants used in the olfactometer experiments are listed in table 1. These plants, as well as lima bean plants *Phaseolus lunatus* L. (Fabaceae), that were used for rearing the spider mite *T. urticae*, were grown in a greenhouse (20-30 °C, r.h. 60-80 % and 16L:8D). Leaves were taken from a grapevine *Vitis vinifera* L. (Vitaceae) grown outdoors (height about 0.5-1 m) and ginkgo leaves from a mature ginkgo tree *Ginkgo biloba* L. (Ginkgoaceae) in the Botanical Garden of Wageningen University (height about 15-20 m). The age of the greenhouse-reared plants and the mean leaf area of the leaves used in the experiments are given in table 1. The leaf area was measured with an area meter (Li-Cor LI-3100, CaTeC, the Netherlands) and the average of ten leaves is presented unless mentioned otherwise. Hop, *Humulus lupulus* L. (Moraceae), was vegetatively propagated from plants grown outdoors and subsequently reared in the greenhouse. Test plants were infested by placing spider mite-infested lima bean leaves on top of their leaves and were kept in a separate compartment in the greenhouse together with the spider mite-infested lima bean plants. Only fully expanded leaves were used for the experiments. In the case of soybean, *Glycine max* L. (Fabaceae), and cowpea, *Vigna unguiculata* L. (Fabaceae), fully expanded trifoliate leaves were used.

Rearing of mites

The spider mites, *T. urticae*, were reared on lima bean under the same conditions as the uninfested lima bean plants. The predatory mites, *P. persimilis*, were originally obtained from Entocare cv, Wageningen, the Netherlands. They were reared in the laboratory on spider mite-infested lima bean leaves or test plant leaves in Petri dishes sealed with parafilm. The Petri dishes were kept in a temperature controlled room (23 ± 2 °C, r.h. 60-80 % and 16L:8D). Fresh leaves

were added every two to three days. For the experiments only young adult females of *P. persimilis* were used, whose age was 1 - 7 days since the final moult.

Olfactometer experiment

The attraction of the predatory mites towards the spider mite-infested leaves was investigated with a closed system Y-tube olfactometer described by Takabayashi & Dicke (1992). Two glass jars of two litres each that contained the odour sources were connected to the Y-tube. Filtered air at 4 l/min was led through each of the jars into the olfactometer. The other side was connected to house vacuum (8 l/min). Before the start of the experiment, the arms of the olfactometer were flushed with air going through the odour sources for about ten minutes. Adult female predatory mites, starved for 1-3 h, were allowed to walk upwind on an iron wire in the Y-tube. At the Y-junction they could make a choice between the two odour streams coming from the two glass jars. When the predatory mite had reached the end of one of the arms her choice was recorded. When she did not reach the end of one of the arms within 5 min, the predatory mite was removed and excluded from statistical analysis.

Table 1: Plants used in olfactometer tests

Family	Genus	Species	Common name	Cultivar	Age (weeks)	Mean leaf area ± SD (cm^2)	% of *T. urticae* that accepted the plant ± SD[4]
Individual plant species tested in an olfactometer							
Fabaceae	*Glycine*	*max*	Soybean	Gieso	5	115 ± 10	99 ± 1 [4]
Fabaceae	*Vigna*	*unguiculata*	Cowpea	Black eye	6	145 ± 25	65 ± 6
Ginkgoaceae	*Ginkgo*	*biloba*	Ginkgo		- [2]	40 ± 5	11 ± 3
Moraceae	*Humulus*	*lupulus*	Hop		16 [1]	20 ± 5 [3]	98 ± 1
Solanaceae	*Capsicum*	*annuum*	Sweet pepper	Lambada	10	90 ± 20	25 ± 5
Solanaceae	*Solanum*	*melalonga*	Eggplant	Black beauty	10	305 ± 45	74 ± 6
Vitaceae	*Vitis*	*vinifera*	Grapevine	Glorie van Boskoop	- [2]	165 ± 20	43 ± 7
Plants tested for attractiveness by predatory mites on the same day							
Fabaceae	*Glycine*	*max*	Soybean	Gieso	4	100 ± 15	
Solanaceae	*Solanum*	*melalonga*	Eggplant	Black beauty	8	260 ± 25	

[1] Age calculated from the time the plant was vegetatively propagated
[2] Age not known
[3] A total of 50 leaves was taken to calculate the average leaf size
[4] Plant acceptance by *Tetranychus urticae* is based on the percentage of spider mites that accepted a leaf section of the test plant when the alternative was a distant leaf section of a lima bean leaf (see Van den Boom et al., 2002 for details)

Spider mite-infested versus uninfested leaves of various plant species

In one glass jar of the olfactometer three clean test plant leaves were placed with their petioles in wet cotton wool. For hop, 9-13 test plant leaves were used, because its leaf area was relatively small. In the other glass jar the same amount of plant leaves infested with spider mites for at least three days and at most 14 days was placed. These sets of infested leaves are considered strong odour sources, as two lima bean leaves that have each been infested for one day by 100 spider mites are highly attractive to *P. persimilis* (Janssen *et al.*, 1997). Even much lower degrees of infestation attract the predators (M. Dicke, unpublished data). Ginkgo is not accepted as a host plant by *T. urticae* and thus, it was not possible to rear spider mites on ginkgo. Therefore, ginkgo leaves were treated with jasmonic acid to mimic the volatile induction profile (Boland *et al.*, 1995). For that purpose 20 ginkgo leaves were individually placed with their petioles into a vial with 10 ml of an aqueous jasmonic acid solution (1 mM) for one day or for three days, respectively. The vials were sealed with parafilm. The uptake of the jasmonic acid solution by the ginkgo leaves was 1 ml a day. As a control for the jasmonic acid solution, 20 ginkgo leaves were placed in 10 ml of an diluted acidic solution (1 mM HCl) during the same time period as the jasmonic acid-treated leaves. All olfactometer experiments for each species were carried out on 3-6 different days. On each day ten predatory mites reared on spider mite-infested lima bean leaves and ten predatory mites reared on spider mite-infested test plant leaves tested were alternately introduced into the olfactometer. For the experiments with soybean (Fabaceae) tested individually and soybean and eggplant (*Solanum melalonga* L. Solanaceae) tested on the same day, only predatory mites reared on spider mite-infested lima bean leaves were used. New odour sources were used every day. For each experiment the odour sources were interchanged after five predatory mites had individually made a choice.

The same mites tested on two plant species on the same day

In the previous experiments all test plant species were investigated on different days and in different weeks. This may make it difficult to compare the relative degree of attraction measured. To evaluate this, two different test plant species were tested with the same predatory mites on the same day. In this setup only the relative differences in odour blends could contribute to differences in predatory mite attraction. Two identical olfactometers were used that were set-up side by side. In one olfactometer six infested versus six uninfested soybean leaves and in the other olfactometer four infested eggplant versus four uninfested eggplant leaves were tested. More soybean than eggplant leaves were tested, because the soybean leaf area was smaller (table 1). The leaves were infested with spider mites for four to seven days. The experiments were carried out on five different days with 20 predatory mites reared on spider mite-infested lima bean per day. Each individual predatory mite was allowed to make a choice in one olfactometer and subsequently in the other olfactometer. After five mites had made a choice in both olfactometers, the order of initial choice for the predatory mites for one out of two olfactometers was changed. After in total ten predatory mites had made their choice in both olfactometers, the infested and uninfested odour sources of one olfactometer were interchanged with the infested and uninfested odour sources of the other olfactometer. After the arms of the olfactometer had been flushed with air coming from the changed odour sources for 10 min., another ten predatory mites could make their choice in both olfactometers.

Statistics

For the predatory mites with the same rearing history, a χ^2-test on the total number of predators attracted towards one of the odour sources was performed per test plant. A 2x2 contingency table was used to analyze whether the attraction per test plant for the predatory mites with different rearing histories was significantly different. To evaluate if the response of the predatory mites towards

the soybean and the response towards eggplant in the olfactometer setup were significantly different, also a 2x2 contingency table was used.

Results

Attraction of predatory mites reared on infested lima bean leaves

Phytoseiulus persimilis females reared on infested lima bean leaves were significantly attracted to *T. urticae*-infested plants of all species tested (figure 1).

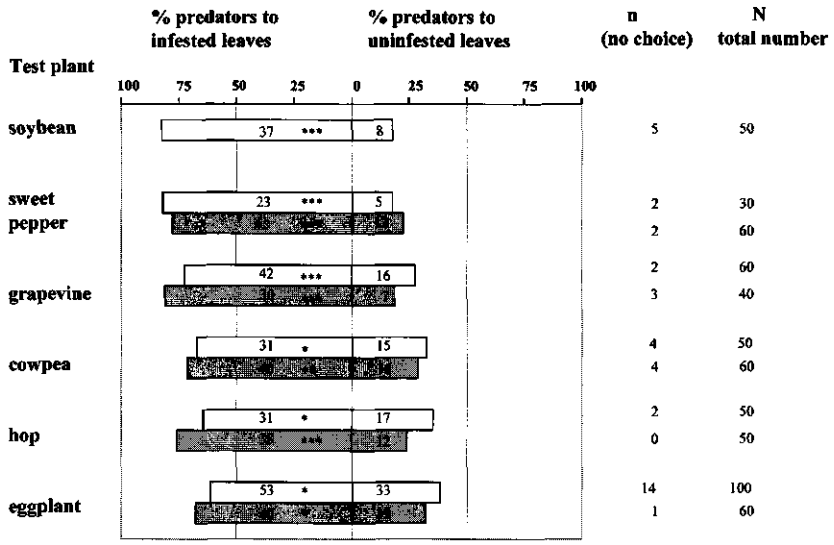

Figure 1: Response of adult female *Phytoseiulus persimilis* towards *Tetranychus urticae*-infested leaves versus uninfested leaves of the same plant species in a Y-tube olfactometer. The predatory mites were reared on spider mites feeding on either lima bean or on the test plant. The number of predatory mites that made a choice to one of the sides is depicted in the bars. The number of predatory mites that did not make a choice (n) and the total number of predatory mites tested (N) is shown at the right side of the figure. Statistics: χ^2-test (*** = $P \leq 0.001$, ** = $0.01 \geq P > 0.001$, * = $0.05 \geq P > 0.01$, n.s. = $P > 0.05$)

□ *P. persimilis* reared on lima bean ■ *P. persimilis* reared on the test plant species

They showed the strongest attraction to infested soybean, sweet pepper (*Capsicum annuum* L. (Solanaceae)) and grapevine, with respectively 82, 82 and 72 % of the predatory mites moving towards the spider mite-infested lima bean

leaves (X^2-test, $P \leq 0.001$). To cowpea, hop and eggplant the response of the predatory mites was weaker, with respectively 67, 65 and 62 % (X^2-test, $0.05 \geq P > 0.01$).

Attraction of predatory mites reared on infested test plant leaves

When the predatory mites were reared on infested leaves of the test plant, the attraction of the predatory mites to the spider mite-infested leaves was also significant for all plant species (figure 1). Strongest attraction was shown by the predatory mites to sweet pepper, grapevine and hop, respectively 78, 81 and 76 % (X^2-test, $P \leq 0.001$). For cowpea 71 % (X^2-test, $0.01 \geq P > 0.001$) and for eggplant only 68 % (X^2-test, $0.05 \geq P > 0.01$) of the predatory mites chose the infested leaves. For none of the test plant species was a significant difference in attractiveness found between predatory mites with different rearing histories.

The same mites tested on two plant species on the same day

For an experiment in which two plant species were compared on the same day, soybean and eggplant were chosen, because they were the two extremes in predatory mite attraction in the separate olfactometer experiments (82 and 62 % respectively, figure 1). Figure 2 shows that there was a significant difference in the response of the predatory mites towards the two plant species (2x2 contingency table, $P = 0.008$).

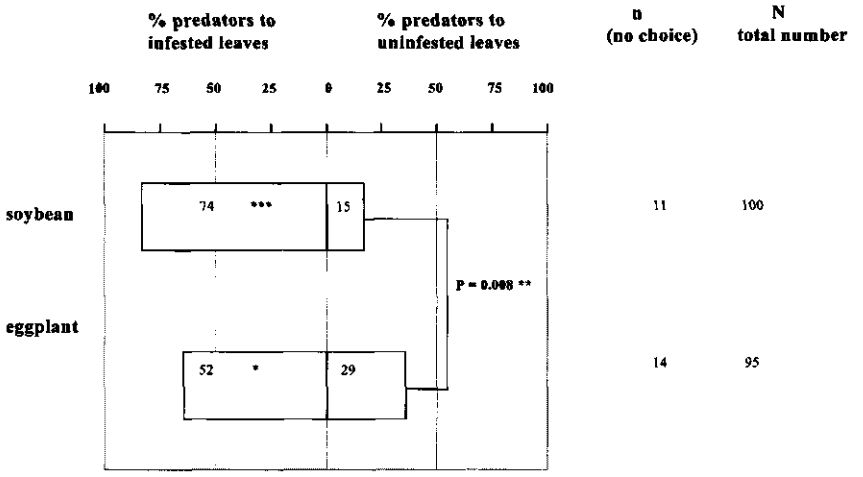

Figure 2: Response of adult female *Phytoseiulus persimilis* reared on lima bean tested in an olfactometer for both soybean and eggplant on the same day. For each test plant *Tetranychus urticae*-infested leaves were tested versus uninfested leaves. For statistics: a χ^2-test and a 2*2 contingency table were used (see figure 1 for an explanation of the asterisks).

For soybean, 83 % of the predatory mites moved towards the spider mite-infested leaves and for eggplant only 64 % of the predatory mites went to the spider mite infested leaves. These predatory mite responses to both infested soybean and infested eggplant do not differ significantly from the responses shown in figure 1.

Attraction of predatory mites by jasmonic acid-induced plant odour

There was no significant attraction of the predatory mites to the jasmonic acid-treated ginkgo leaves (figure 3) after one day or after three days of incubation. Respectively 60 % and 59 % of the predatory mites went to the jasmonic acid-treated leaves. The responses of the predatory mites towards the jasmonic acid-treated ginkgo leaves with different incubation times were not significantly

different, and therefore the data were pooled. The pooled data revealed a significant attraction ($P < 0.05$) of the predatory mites to jasmonic acid-treated ginkgo leaves (figure 3).

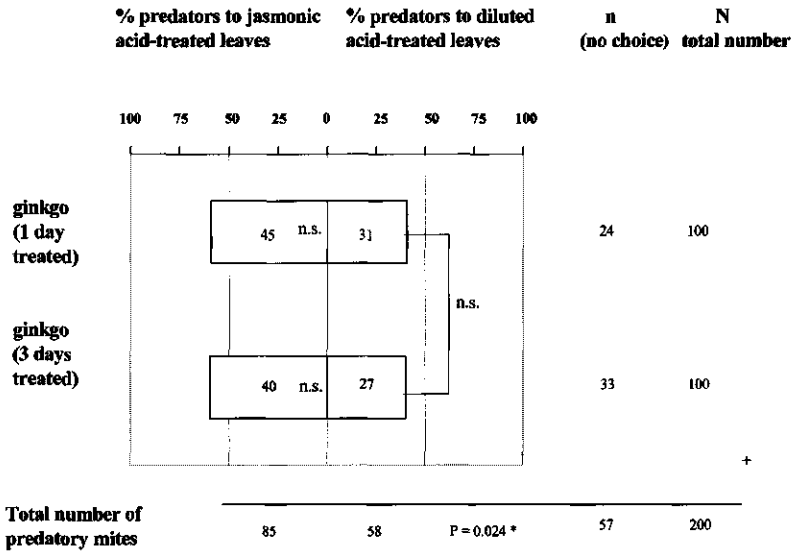

Figure 3: The attraction of *Phytoseiulus persimilis* towards jasmonic acid-treated ginkgo leaves versus hydrochloric acid-treated ginkgo leaves. The leaf petioles were placed in an aqueous jasmonic acid solution and the control leaf petioles in a diluted acidic solution during one day or during three days. The pooled data with the total number of predatory mites are also depicted. For statistics: see figure 1.

Discussion

Attraction of predatory mites reared on infested lima bean leaves

The *T. urticae*-infested plants of all species tested in the olfactometer produced volatiles that attract the predatory mite *P. persimilis*. The components that play a role in the attraction of predatory mites to spider mite-induced plants have been investigated for lima bean (Dicke *et al.*, 1990a). The induced components from lima bean that individually attracted the predatory mites were (3*E*)-4,8-dimethyl-1,3,7-nonatriene, linalool, (*E*)-ß-ocimene and methyl salicylate. It is possible that one or more of these attractive components were also released by the currently investigated plants. It is known that beet armyworm, *Spodoptera exigua*, induces (3*E*)-4,8-dimethyl-1,3,7-nonatriene in cowpea leaves, but in soybean leaves this component is not induced or only in very small concentrations (Turlings *et al.*, 1993). Furthermore, it has been shown that methyl salicylate is induced by spring migrants of the damson-hop aphid, *Phorodon humuli* Schrank (Homoptera, Aphididae), when they feed on hop leaves (Campbell *et al.*, 1993). When different herbivores feed on the same host plant species, the volatile blends are generally similar in qualitative sense, though quantitative differences in the relative contributions of the blend components occur (see Turlings *et al.*, 1993 and Dicke, 1999b for reviews).

Attraction of predatory mites reared on infested test plant leaves

The predators were attracted to *T. urticae*-induced volatiles of all test plant species independent of rearing history. This has been documented in more cases where predatory mites were attracted to spider mite-infested test plants without experience on these plant species (Dicke & Sabelis, 1988a). However, for gerbera and cucumber the predatory mites were only attracted to the spider mite-infested plant leaves when reared on spider mites on these plants (Dicke *et al.*, 1990b; Krips *et al.*, 1999). Both spider mite-infested gerbera and cucumber

induced the compounds (3*E*)-4,8-dimethyl-1,3,7-nonatriene, (*E*)-ß-ocimene and small amounts of linalool (Takabayashi *et al.*, 1994b; Krips *et al.*, 1999). Methyl salicylate was only induced in minor amounts in gerbera. Associative learning was proven for the predatory mite *P. persimilis* by Drukker *et al.* (2000). They showed that naïve predatory mites responded positively to odour from an unfamiliar environment when a positive experience with prey in combination with the investigated odour source was given to the predatory mites.

The same mites tested on two plant species on the same day

Even though more leaves were used for both soybean and eggplant than in the separate experiments, the predatory mites showed the same percentage of response to each of the plants. Also the relative response of the predatory mites towards infested eggplant and soybean leaves was not significantly different. Thus, there is no evidence that our data on the relative attraction to different plant species have been affected by a day-effect or by variation in the number of infested leaves.

Attraction of predatory mites by jasmonic acid-induced plant odour

The pooled data of the two jasmonic acid-experiments that differ in incubation time show that the predatory mites were slightly attracted to the jasmonic acid-induced ginkgo leaves. Although jasmonic acid can mimic the spider mite-induced blend, the two blends are not identical. Dicke *et al.* (1999) used lima bean to investigate the differences in the jasmonic acid- and spider mite-induced volatiles and concluded that most of the jasmonic acid-induced components were the same as the spider mite induced components. However, some components were not produced in response to jasmonic acid application or only in smaller quantities. Dicke *et al.* (1999) also found that predatory mites were attracted to the jasmonic acid-induced lima bean leaves, but the predators preferred the spider mite-induced lima bean leaves in a 2-choice test. Boland *et al.* (1995)

determined the components that were induced when 10 mM jasmonic acid was supplied through the petiole of freshly detached ginkgo leaf for 30 hours and found that linalool was induced in large quantities and methyl salicylate in low quantities. The components (3*E*)-4,8-dimethyl-1,3,7-nonatriene and (*E*)-ß-ocimene were not induced at all. A slight but significant predatory mite attraction to the jasmonic acid-treated ginkgo leaves was found, possibly due to the induction of linalool and small amounts of methyl salicylate. Although linalool and methyl salicylate can individually attract predatory mites at certain concentrations (Dicke *et al.*, 1990a), it might be possible that their induction was not sufficient to give a strong predatory mite response.

Direct versus indirect defence in a plant species

It was hypothesized that plants with a strong direct defence have a less efficient indirect defence than plants that are not characterized by a strong direct defence. Ginkgo has a strong direct defence mechanism against spider mites (Dabrowski, 1973). Therefore, it is not really necessary for ginkgo to induce synomones for the attraction of predatory mites. However, a slight but significant attraction of the predatory mites to the jasmonic acid-treated leaves was found. This indicates that indirect defence exists in jasmonic acid-treated ginkgo to a minor extent. This effect may even have been stronger if it would be possible to induce the leaves through spider mite-feeding. Soybean and hop were highly susceptible to spider mites in contrast to sweet pepper and grapevine, which were poorly accepted by spider mites (van den Boom *et al.*, 2003). However, spider mite-infested leaves of all these plants were highly attractive to predatory mites. Eggplant and cowpea were less susceptible to spider mites than soybean and hop (van den Boom *et al.*, 2003). Spider mite-infested leaves of these plants showed a less prominent predatory mite attraction, although their leaves were quite heavily infested. It might be interesting to use more than three leaves in the olfactometer experiments, although Janssen *et al.* (1997) showed that predatory mites were

already attracted by only two lima bean leaves each infested with one hundred *T. urticae* females.

In conclusion, *T.urticae*-infested plants of all species tested, emitted induced synomones that attracted the predatory mites. Even jasmonic acid-treated ginkgo leaves that possess a strong direct defence were slightly attractive to predatory mites. Furthermore, both among the Fabaceae (soybean and cowpea) and among the Solanaceae (sweet pepper and eggplant), plant species use indirect defence to defend themselves. However, it remains to be investigated whether all these plant species have a similar mechanism of indirect defence. For instance, plants may produce novel compounds in response to herbivory, which results in a specific blend composition or they may emit a qualitatively similar blend that differs in ratios among blend components (Dicke, 1999b). The emission of novel compounds represents the induction of novel biosynthetic pathways (Paré & Tumlinson 1997, Dicke 1999b) and may be seen as a more sophisticated form of indirect defence than the emission of volatiles through pathways that are also induced by mechanical damage. The type of induced odour production of the plant species tested is currently being examined. The relationship between the specificity of indirect defense and the degree of a plant's direct defense is currently being investigated.

Acknowledgements

The authors would like to thank Herman Dijkman for the supply of spider mites. Henk Smid took care of the plants in the greenhouse. Furthermore we thank the PhD discussion group of the laboratory of Entomology for their comments on a previous version of the manuscript.

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Qualitative and quantitative variation among volatile profiles induced by *Tetranychus urticae* feeding on plants from various families

Abstract

Many plant species are known to emit herbivore-induced volatiles in response to herbivory. This has been studied for different plant-herbivore combinations. The spider mite *Tetranychus urticae* Koch is a generalist that can feed on several hundreds of host plant species. Here, the volatiles emitted by *T. urticae*-infested plants of eleven species were compared. First, the degree to which the plant species produced novel compounds was analyzed when compared to the odors of mechanically damaged leaves. Almost all of the investigated plant species produced novel compounds that dominated the volatile blend, such as methyl salicylate, terpenes, oximes and nitriles. Only spider mite-infested eggplant and tobacco emitted a blend that was merely quantitatively different from the blend emitted by mechanically damaged or clean leaves. We hypothesized that plant species with a low degree of direct defense would produce more novel compounds. Novel compounds might be more reliable cues for predatory mites to find their prey. However, it was concluded that although plant species with a low direct defense level do use indirect defense to defend themselves, they do not always emit novel compounds. Plant species with a high level of direct defense seem to invest in the production of novel compounds. When plant species of the Fabaceae were compared to plant species of the Solanaceae, qualitative differences in spider mite-induced volatile blends seem to be more prominent in the Fabaceae than in the Solanaceae.

This Chapter was submitted to the Journal of Chemical Ecoloy

Introduction

Infochemicals are important mediators of tritrophic interactions such as predator-herbivore-plant interactions or parasitoid-herbivore-plant interactions. Behavioral and chemical studies have proven that carnivorous arthropods, such as predators and parasitoids, can be attracted to plant volatiles that are induced by their prey or hosts (for example: Turlings et al. 1995; Agelopoulos and Keller, 1994; Blaakmeer et al., 1994; Dicke, 1999a; Dicke et al., 1998). Moreover, the carnivores are often not or to a lesser extent attracted to volatiles from mechanically damaged leaves, or to volatiles from leaves infested by herbivores that are not suitable as prey or hosts (Dicke, 1999a; Dicke and Sabelis, 1988a; Du et al., 1998; Guerrieri et al., 1999; de Moraes et al., 1998; Shimoda and Dicke, 2000; Sabelis and van den Baan, 1983; Vet and Dicke, 1992). The infochemicals involved in such interactions are called synomones, which means that they are beneficial for both emitter and receiver (Dicke and Sabelis, 1988b). That infochemicals can be beneficial for the plant (emitter) has been shown for *Pieris rapae* caterpillars feeding on *Arabidopsis thaliana* after being parasitized by *Cotesia rubecula* feeding on these plants (van Loon et al., 2000). Also for *Zea mays* an increase in seed production has been recorded after *Spodoptera littoralis* caterpillar larvae, that were feeding on its leaves, had been parasitized by *Cotesia marginiventris* or *Campoletis sonorensis* (Fritzsche-Hoballah and Turlings, 2001).

The volatile profiles emitted by herbivore-infested conspecific plants vary when different herbivore species are feeding on them. This variation in volatile profiles is important for carnivores to find their prey. For example, predatory mites can distinguish between volatiles induced by damage inflicted by different prey mite species (Dicke and Sabelis, 1988a; Dicke et al., 1988; Sabelis and van den Baan, 1983; Sabelis and Dicke, 1985; Takabayashi et al., 1991). Moreover, a stronger degree of variation exists among volatile profiles when a herbivore species is

feeding on plants from different species (Dicke, 1999a; Takabayashi and Dicke, 1996; Turlings et al., 1993; Takabayashi et al., 1994a). The herbivore-induced volatile profile can differ qualitatively or quantitatively from the profile emitted in response to mechanical damage. Dicke et al. (1998) discriminated two types of volatile profiles emitted from herbivore-infested plants. The first type of a volatile profile is qualitatively different from the blend emitted by mechanically damaged leaves, with the emission of novel compounds that make a major contribution to the total blend. The emission of novel compounds in the qualitatively different volatile blend represents the induction of novel biosynthetic pathways (Dicke, 1999b; Paré and Tumlinson, 1997). This can be considered a more sophisticated form of indirect defense than the emission of volatiles through pathways that are also induced by mechanical damage. The second type of a volatile profile is quantitatively different from that emitted by mechanically damaged leaves. In herbivore-infested leaves larger quantities are emitted of compounds that are also produced by mechanically damaged leaves. In addition, the compounds are emitted from the herbivore-infested plant for a longer time than from mechanically damaged plants (Steinberg et al., 1993).

After herbivore damage, different plant species can attract predators or parasitoids from a distance through the emission of volatiles. To be able to do so, the detectability of the induced odor blend is of great importance for the carnivore (Vet and Dicke, 1992). The production of large quantities of volatiles can increase the detectability of the odor source, especially when these volatiles have low boiling points. On a shorter range, when the predators or parasitoids are closer to their prey or hosts, they can be arrested by the volatiles in and around a prey patch (Dicke et al., 1998). An important aspect of the odor blend for the predator or parasitoid is whether it provides reliable information on herbivore presence and identity. The production of novel compounds that dominate the volatile blend can be a reliable indicator of herbivore presence and potentially

also its identity to the predators or parasitoids. In contrast, it is important for the herbivores to reduce their odor induction as much as possible, so they may benefit from selecting host plants that do not produce a blend which might attract their carnivorous enemies (Sabelis and Dicke, 1985; Turlings et al., 1993).

Plant species that have a strong direct defense may have no ecological need to invest in indirect defense through the emission of specific volatiles. These plant species might induce more compounds that contribute to the degree of a plant's direct defense and in the meantime quantitatively increase their volatile emission profile to attract predatory mites. However, plant species that do not have a strong direct defense may rely on an effective indirect defense through specific volatiles that provide predatory mites with reliable information. However, it is not known to what degree these two different defense mechanisms are compatible and to what extent a plant can bear the costs to use both types of defenses. A trade-off between the level of direct and indirect plant defense has been shown for *Nicotiana attenuata* Torr. Ex. Watson (Kahl *et al.*, 2000). When *Manduca sexta* (Lepidoptera: Sphingidae) larvae feed on *N. attenuata* these plants increase the release of volatile terpenoids (indirect defense), but they do not increase the levels of nicotine (direct defense). In contrast, both types of defense are induced simultaneously in tomato, albeit that the induced indirect defense does not consist of major novel compounds (Dicke et al., 1998, Thaler et al., 2002).

For the spider mite *Tetranychus urticae* and its natural enemy, the predatory mite *Phytoseiulus persimilis*, it has been shown that the predatory mite is attracted by spider mite-induced compounds in volatile blends of several plant species. The investigated plant species comprise lima bean (Dicke et al., 1990a), apple (Takabayashi et al., 1991), cucumber (Takabayashi et al., 1994b), tomato (Dicke et al., 1998) and gerbera (Krips et al., 1999) plants. Both lima bean and cucumber are examples of plant species that emit a qualitatively different blend in response

to spider mite damage compared to mechanical damage (Dicke, 1994; Dicke et al., 1990a; Takabayashi et al., 1994b). Tomato is an example of a plant species that emits a blend in response to spider mite feeding that is only quantitatively different from the blend of mechanically damaged plants, because there are no novel volatiles emitted from tomato after spider mite damage that dominate the blend (Dicke et al., 1998).

The degree of direct defense of several plant species from various families to the spider mite *T. urticae* has been investigated (van den Boom et al., 2003) as well as their capability to induce indirect defense, i.e. their ability to attract the spider mite's natural enemy *P. persimilis* (van den Boom et al., 2002). To obtain more information on the mechanism of the induced indirect defense, the identity of the volatiles that are released by plants from various species when *T. urticae* is feeding on these plant species was investigated and compared to the volatiles emitted from uninfested and mechanically damaged leaves. In this way it can be revealed whether major novel biosynthetic pathways are induced by the spider mite's feeding.

Special attention has been paid to plant species of the Fabaceae and the Solanaceae. These plant families varied in their degree of direct defense (van den Boom et al., 2003). All investigated plant species of the Fabaceae were readily accepted for feeding by the spider mite *T. urticae*, while the investigated plant species of the Solanaceae showed more variation with respect to spider mite acceptance, from being well accepted to being poorly accepted. Some plant species from the Fabaceae have already been investigated and headspace analyses have shown that they emit novel volatiles in response to herbivory that are not emitted from mechanically damaged or undamaged plants. This has been shown for spider mite-infested lima bean (Dicke et al., 1990a) and aphid-infested broad bean that attracts the parasitic wasp *Aphidius ervi* (Du et al., 1998).

Headspace analyses with plant species from the Solanaceae such as potato infested with larvae of the Colorado potato beetle (Bolter et al., 1997) and tomato infested with the spider mite *T. urticae* (Dicke et al., 1998) have revealed that they do not emit novel volatiles. Differences in the type of volatile production can elucidate if certain plants or plant families possess a more sophisticated way of indirect defense than others.

Materials and methods

Plant material

The plant species used are listed in Table 1. The plants were grown in a greenhouse (20-30 °C, r.h. 60-80 % and 16L:8D). Lima bean plants (*Phaseolus lunatus* L.) that were used as host plants for the spider mites were reared under the same conditions. Hop plants were vegetatively propagated from plants grown outdoors and subsequently reared in the greenhouse. The grapevine (height: about 0.5-1 m) grew outdoors in Wageningen, as well as several black locust trees (height: 1-1.5 m), golden chain trees (height: 1-1.5 m) and a ginkgo tree (height: about 15-20 m) in the Arboretum of Wageningen University. The age of the plants and trees and their mean leaf area are given in Table 1. The mean leaf area was measured with an area meter (Li-Cor LI-3100) and is the average of 10 leaves unless mentioned otherwise. Test plants and trees (except the ginkgo tree) were kept per species in a separate compartment in the greenhouse. They were infested by placing spider mite-infested lima bean leaves on top of their leaves Only fully expanded leaves were used for the experiments. In the case of soybean and cowpea fully expanded trifoliar leaves were used.

Rearing of mites

The spider mites, *Tetranychus urticae*, were reared on lima bean plants under the same conditions as the uninfested lima bean plants. This was done in a separate greenhouse compartment.

Table 1A: Plants and trees used in the headspace experiments.

Family	Genus	Species	Common name	Cultivar
Fabaceae	*Glycine*	*max*	soybean	Gieso
Fabaceae	*Laburnum*	*anagyroides*	golden chain	
Fabaceae	*Robinia*	*pseudo-acacia*	black locust	
Fabaceae	*Vigna*	*unguiculata*	cowpea	Black eye
Ginkgoaceae	*Ginkgo*	*biloba*	ginkgo	
Moraceae	*Humulus*	*lupulus*	hop	
Solanaceae	*Capsicum*	*annuum*	sweet pepper	Lambada
Solanaceae	*Datura*	*stramonium*	thorn apple	
Solanaceae	*Nicotiana*	*tabacum*	tobacco	SR1
Solanaceae	*Solanum*	*melalonga*	eggplant	Black beauty
Vitaceae	*Vitis*	*vinifera*	grapevine	Glorie van Boskoop

Table 1B: Information on leaves used in the headspace experiments.

Common Name	Age (weeks)	Number of leaves used	Mean leaf area ± SD (cm^2)	Spider mite damage (days)
soybean	5	2	115 ± 10	8
golden chain	- [1]	10	20 ± 5 [3]	3
black locust	- [1]	50	15 ± 5 [3]	4
cowpea	4	2	160 ± 25	8
ginkgo	- [1]	5	40 ± 5	- [4]
hop	25 [2]	3	65 ± 5 [3]	8
sweet pepper	12	3	120 ± 20	6
thorn apple	6	3	115 ± 25	8
tobacco	8	3	125 ± 35	7
eggplant	8	2	260 ± 25	7
grapevine	- [1]	2	165 ± 20	8

[1] Age not known, but ≥ 3 years

[2] Age calculated from the time the plant was vegetatively propagated

[3] An average of 50 leaves was taken to calculate the average leaf size

[4] No spider mite-infestation but jasmonic acid used for volatile-induction

Setup for headspace collection

A method was used to sample the volatiles from six 5 liter-glass jars connected in parallel at the same time. Synthetic air (grade 5.0) was led via Teflon tubing over an activated charcoal filter and a molecular sieve (5A). Via a glass connection the air was divided equally over the six glass jars. The air entered the jars via the top and left the jar via a glass tube (160 mm long x 6.0 mm OD, 4 mm ID) that was connected to the air outlet at the bottom of the jar. This tube was filled with 90 ± 10 mg Tenax-TA (20-35 mesh, 500-840 μm) and kept in place by two stainless steel frits. Prior to headspace collection, Tenax tubes were cleaned in a Thermal Desorption Oven (TDS, Gerstel, The Netherlands) at 250 °C. A flow meter was used to measure the flow rate at the end of the tubes filled with Tenax. The flow rate was kept at 100 ± 20 ml/min during the experiments. Fresh test plant leaves were cut and placed with their petioles into a thick layer of wet cotton wool in five of the glass jars, to obtain five replicate measurements. Plant volatiles were sampled for 2 hours. For most plant species two or three test plant leaves were used, but for ginkgo and golden chain more leaves were used, i.e. 5 and 10 leaves respectively, because these leaves were much smaller than the leaves of the other plant species (Table 1). For black locust 5 small branches of 10 leaves were used for the experiments. The sixth glass jar contained only a piece of wet cotton wool and the air sampled from this jar was used as a blank.

Besides the parallel headspace setup, also one 5-liter glass jar with two Tenax tubes at opposite outlets was used. The flow rate through both tubes was kept at 100 ± 20 ml/min. In this way two replicate headspace samples from the same set of plant leaves were obtained. One tube of the two twin replicates was desorbed in a thermal desorption system (TDS) connected to an autosampler (TDS-A) and the compounds were analyzed on an RTX-200 column in a gas chromatograph (HP5890) and detected with a flame ionisation detector (FID) (see the paragraph 'gas chromatography' for a more detailed description). The same procedure was

followed for the other five tubes that had been obtained from the parallel headspace experiment. The other tube of the two twin replicates was desorbed in a TDS as well and the compounds were analyzed on the same type of column in a gas chromatograph connected to a mass spectrometer (GC-MS; Varian 3400). A Finnigan MAT 95 mass spectrometer was used for detection of the compounds (see the paragraph 'gas chromatography and mass spectrometry' for a more detailed description). After the GC-MS analysis, the compounds were identified by comparison of their retention indices and mass spectra to reference spectra in the NIST and the natural products library of the mass spectrometry section of Wageningen University. Subsequently, the GC-MS chromatogram with the identified peaks was compared with the gas chromatogram obtained from the twin-replicate Tenax tube that had been analyzed with an FID, by comparing the peak shape and the retention times of the peaks. When the retention times of the peaks in the gas chromatogram were known, they could be compared to the retention times of the compounds in the five replicate chromatograms obtained from the parallel headspace measurements, that were measured with the same apparatus and on the same column.

Gas Chromatography

The volatiles adsorbed on the Tenax in the headspace tube were desorbed in a splitless mode in a TDS with helium (grade 6.0). The temperature of the TDS was kept at 30 °C for 0.5 min and was subsequently increased at a rate of 60 °C/min to 250 °C. Desorbed compounds were transferred via a heated transfer line (300 °C) into the empty liner of a gas chromatograph (HP 5890). In the injector the volatiles were trapped again at –150 °C. After trapping of the volatiles, the injector was heated at a rate of 12 °C/sec up to 250 °C. The volatiles were transferred in splitless mode onto an uncoated deactivated fused silica precolumn that was split into two columns, an RTX-200 column (Restek corporation: 60 m long, 0.25 mm ID, 0.25 μm film thickness) and a non-polar

AT-1 column (Alltech: 60 m long, 0.25 mm ID, 0.25 µm film thickness). Two columns of different polarity were chosen to obtain quantitative reference values in order to resolve peaks that co-eluted. The GC oven temperature was first kept for 3 min at 40 °C and then increased with a rate of 4 °C/min to 240 °C. An FID was used to detect the volatile compounds. Chromatograms were obtained for five headspace collections of each treatment and one blank.

Gas Chromatography and Mass Spectrometry

A gas chromatograph (Varian 3400) connected to a Finnigan MAT 95 mass spectrometer was used to identify the headspace compounds collected on the Tenax tubes. The volatiles were thermally desorbed in splitless mode with helium as carrier gas at a temperature of 250 °C and subsequently cryofocused in a Thermal Desorption Cold Trap Injector (M-16200, Chrompack, The Netherlands) at a temperature of –90 °C. The Cold Trap Injector was ballistically heated to 220 °C and the volatiles were transferred in a splitless mode onto an RTX-200 column (Restek: 60 m long, 0.25 mm ID, 0.25 µm film thickness). The oven temperature was first kept for 2 min at 40 °C and then increased at a rate of 4 °C/min to 250 °C. The mass spectrometer was operated in the 70 eV EI ionisation mode. Spectra were continuously scanned in a mass range from 24 to 300 amu at a rate of 0.5 sec/decade.

Leaf treatments

For each test plant species both spider mite-infested, mechanically damaged and clean leaves were used for the headspace analyses. Headspace was collected from each of these treatments in five replicates for each test plant species. After each collection the glass jars were washed and cleaned at 110 °C. The leaves were freshly cut from the plant and headspace was collected immediately. Spider mite-infested leaves were obtained from test plants on which spider mites had been feeding for 3 to 8 days (Table 1). Leaves were mechanically damaged with

carborandum on a wet cotton wool pad after cutting the leaf and headspace was collected just after inflicting the damage. On each leaf a damaged spot of about one by one cm was created.

It was not possible to rear spider mites on ginkgo, because ginkgo was not accepted as host plant by *T. urticae*. Therefore, these leaves were treated with jasmonic acid to simulate spider mite infestation (Boland et al., 1995; Dicke et al. 1999). For that purpose 5 sets of 5 ginkgo leaves were placed with their petioles into vials with 10 ml of an aqueous jasmonic acid solution (1 mM) for 1 day. The vials were sealed with parafilm. The uptake of the jasmonic acid solution by the ginkgo leaves was 1-2 ml after 1 day. As a control for the jasmonic acid solution, 5 sets of 5 leaves were placed for 1 day in vials with 10 ml of a diluted acidic solution (1 mM HCl).

Statistics

The total amount of volatiles produced by the non-infested, mechanically damaged and spider mite-infested leaves were tested with the Kruskal Wallis test followed by a multiple comparison test. Also the relative amounts of compounds were compared among the different treatments of the same test plant species by using the Kruskal Wallis test followed by a multiple comparison test.

Results

The compounds that represented at least 0.5 % of the total amount of the volatile blend emitted by clean, mechanically damaged or spider mite-infested plant leaves are depicted in the figures (Figures 1a-1k).

Spider mite-induced compounds of plants from the Fabaceae

When infested by the spider mite *T. urticae*, all fabaceous species produced compounds that were not or only in small amounts released by mechanically damaged or clean leaves (Figures 1a-1d). Besides, they all released the green leaf volatiles (*Z*)-3-hexen-1-ol (36) and (*Z*)-3-hexen-1-ol, acetate (50) when mechanically damaged. These volatiles were also emitted when the plants were infested by spider mites, but in lower proportions.

Golden Chain (Laburnum anagyroides)

Spider mite-infested leaves from golden chain emitted (*E*,*E*)-α-farnesene (71) as the dominant compound in the volatile blend. This compound is present in significantly larger concentrations in the headspace from spider mite-infested leaves than in the headspace from mechanically damaged and clean leaves: 47 %, 7 % and 8 % respectively. This means that in absolute amounts (*E*,*E*)-α-farnesene (71) is emitted in seventy-fold larger amounts from spider mite-infested leaves than from mechanically damaged leaves. Additionally, novel compounds are present in the spider mite-induced blend. The compounds that are most abundant are 2-methylbutanenitrile (8) (3 %), (syn) or (anti)-2-methylbutanal oxime (12) (8 %), 3-methyl-1-butanol (32) (5 %) and α-humulene (75) (4 %).

Soybean (Glycine max)

Spider mite-infested soybean leaves emitted methyl salicylate (52) as the dominant compound in the blend (13 %). This percentage was statistically different from the percentage in the headspace of mechanically damaged leaves. In absolute amounts more than fifty times as much methyl salicylate is produced by spider mite-infested as by mechanically damaged soybean leaves. (*E*,*E*)-α-

Farnesene (71) was emitted in relatively lower proportions by spider mite-infested as by mechanically damaged soybean leaves (5 % and 12 % respectively) but in absolute amounts twice as much (*E*,*E*)-α-farnesene (71) was produced by spider mite-infested leaves.

Cowpea (Vigna unguiculata)

Cowpea showed a very abundant production of (3*E*)-4,8-dimethyl-1,3,7-nonatriene (63) (74 % of the total amount of volatiles) in response to spider mite damage. This percentage is statistically different from the percentage in the headspace of mechanically damaged leaves in both relative and absolute amounts. Additionally, methyl salicylate (52) and (*E*)-β-farnesene (72) (7 % and 3 % respectively) and some minor compounds were emitted as novel compounds by the spider mite-infested cowpea leaves.

Black locust (Robinia pseudo-acacia)

Black locust emitted several novel nitrogen-containing compounds that dominated the blend, such as three nitriles (8, 9, 18) and nine (O-methyl)-oximes (10-17, 20). Furthermore, (*E*)-β-ocimene (61) (15 %) and some other minor compounds were emitted as novel compounds by spider mite-infested leaves.

Spider mite-induced compounds of plants from the Solanaceae

Two solanaceous species, sweet pepper and thorn apple, produced large amounts of novel compounds when infested by spider mites. The other two plant species, eggplant and tobacco, produced large amounts of compounds that were also released by plants without damage or by mechanically damaged plants (Figure 1e-1h). Besides the non-specific compounds, the headspace of spider mite-infested tobacco leaves also showed minor quantities of novel compounds. All these plants released the green leaf volatiles (*Z*)-3-hexen-1-ol (36) and (*Z*)-3-hexen-1-ol, acetate (50) when mechanically damaged.

Tobacco (Nicotiana tabacum)

Spider mite-infested tobacco leaves emitted non-specific compounds in nearly the same percentages as non-damaged leaves. The percentages for mechanically damaged leaves are much lower, due to a high amount of (*Z*)-3-hexen-1-ol (36) (72 %). The non-specific compounds emitted by spider mite-infested leaves were 1-hexanol (37) (3 %), methyl salicylate (52) (18 %), β-caryophyllene (66) (11 %) and caryophyllene oxide (82) (8 %). Moreover, hexanal (6) (4 %), linalool (43) (6 %), and a few compounds that were produced in small quantities were emitted as novel compounds from the spider mite-infested leaves.

Sweet pepper (Capsicum anuum)

Spider mite-infested sweet pepper emitted three compounds in large amounts that were not or only in small amounts emitted from mechanically damaged leaves, namely the terpenes (*E*)-β-elemene (68) (19 %), germacrene A (73) (37 %) and (3*E*,7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (81) (27 %). Sweet pepper also released the green leaf volatile (*Z*)-3-hexen-1-ol, butanoate (54) in large amounts from both clean and mechanically damaged leaves (both 40 %). Besides, relatively small amounts of 2-butanone (21), linalool (43), methyl salicylate (52), (*E*)-β-ocimene (61), (3*E*)-4,8-dimethyl-1,3,7-nonatriene (63), (*Z*)-β-elemene (69), α-selinene (77) and β-selinene (78) were produced by the spider mite-infested leaves. In absolute amounts however, they are not negligible.

Thorn apple (Datura stramonium)

Spider mite-infested thorn apple produced five novel compounds that made up at least 5 % of the total headspace blend, i.e. 2-butanone (21) (5 %), methyl salicylate (52) (7 %), β-caryophyllene (66) (29 %), (3*E*,7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (81) (33 %) and caryophyllene oxide (82) (7 %). The compound (3*E*,7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (81) is also produced in large amounts by non-damaged leaves. Furthermore, small amounts of the novel compounds (*E*)-β-elemene (68), α-humulene (75), α-selinene (77) and (3*E*,7*Z*) or (3*Z*,7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (80) were emitted.

Eggplant (Solanum melalonga)

Spider mite-infested eggplant leaves emitted non-specific compounds in nearly the same ratios as clean leaves and mechanically damaged leaves. These compounds were 2-methylbutanal-O-methyl oxime (16) (13 %), (3*E*)-4,8-dimethyl-1,3,7-nonatriene (63) (52 %), and α-bergamotene (65) (7 %). However, in absolute amounts the spider mite-infested leaves produced tenfold larger amounts of these compounds than undamaged or mechanically damaged leaves. Furthermore, some small amounts of novel compounds were emitted: 2-methylpropanal-O-methyl oxime (11), 3-methylbutanal-O-methyl oxime (17), (*E*)-β-ocimene (61), (*E*)-β-elemene (68) and (3*E*,7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (81).

Spider mite-induced compounds of plants from other families

The spider mite-infested plants from species of the other families each emitted two or more compounds that dominated the blend (Figure 1i-1k). Both hop and grapevine released the green leaf volatiles (*Z*)-3-hexen-1-ol (36) and (*Z*)-3-hexen-1-ol, acetate (50) when mechanically damaged. Ginkgo did not release any green leaf volatiles when treated with 1 mM jasmonic acid or the control (1 mM HCl) solution.

Hop (Humulus lupulus)

In hop two major novel compounds were emitted from the spider mite-infested leaves namely methyl salicylate (52) (43 %), that is only induced in very small amounts in mechanically damaged leaves and (*E*,*E*)-α-farnesene (71) (34 %). Furthermore, small amounts of 2-methylbutanenitrile (8), 3-methylbutanenitrile (9), β-caryophyllene (66), (*Z*,*E*) or (*E*,*Z*)-α-farnesene (70) and (3*E*,7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (81) were found in the spider mite-infested blend.

Grapevine (Vitis vinifera)

Spider mite-infested grapevine leaves released several novel compounds that dominated the blend. These compounds are methyl salicylate (52) (8 %), (3*E*)-4,8-dimethyl-1,3,7-nonatriene (63) (37 %), β-caryophyllene (66) (11 %) and α-humulene (75) (12 %). In the headspace of spider mite-infested grapevine leaves (3*E*)-4,8-dimethyl-1,3,7-nonatriene (63) occurred in 4-fold higher concentrations (37%) as in the headspace of uninfested leaves (9 %). In absolute amounts the compound was produced about 450 times more abundantly relative to uninfested leaves.

Ginkgo (Ginkgo biloba)

Neither undamaged leaves, nor HCl-treated leaves ginkgo produced many volatiles. However, when treated with 1 mM jasmonic acid-solution, large amounts of volatiles were released. The most dominant compound in the blend was (*E*,*E*)-α-farnesene (71) (57 %). This compound is also present in high concentrations in the headspace of both undamaged and HCl-treated leaves. However, the effect of jasmonic acid on the absolute amount of (*E*,*E*)-α-farnesene (71) emitted was very strong. Other novel compounds that were emitted by JA-treated leaves were eugenol (42) (7 %), linalool (43) (5 %), α-pinene (62) (3 %), β-caryophyllene (66) (10 %), α-copaene (67) (13 %), (*Z*,*E*) or (*E*,*Z*)-α-farnesene (70) (3 %), and γ-selinene (79) (1 %). Limonene (60) was produced in higher percentages in HCl-treated leaves (79 %) than in jasmonic acid-treated leaves (2 %). In absolute amounts limonene was present in twofold larger amounts in the blend emitted by the HCl-treated leaves.

Variation in release of plant volatiles

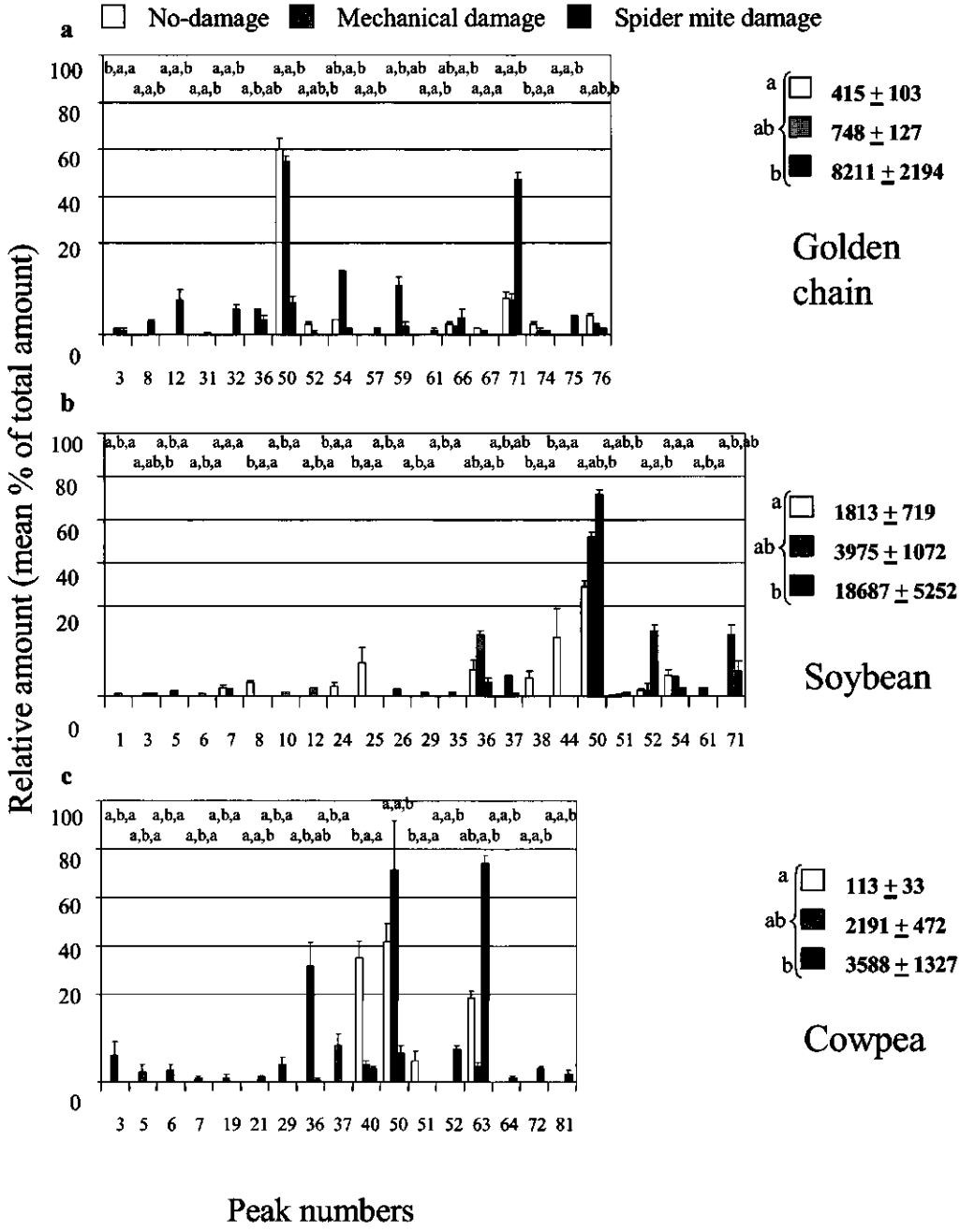
Uninfested plants from all plant species except thorn apple and hop released a statistically significant smaller amount of volatiles ($P < 0.05$) compared to spider mite-infested plants (Figures 1a-1f and 1h-1k). For thorn apple and hop (Figure 1g and 1i) the amount of volatiles from uninfested leaves was significantly smaller than the amount released from mechanically damaged leaves but not

compared to the amount released from spider mite-infested leaves. However, the amount of volatiles from spider mite-infested leaves was of the same order as the amount from mechanically damaged leaves.

Figure 1a – 1k. The plant compounds present at 0.5 % or higher concentrations in the headspace of clean leaves, mechanically damaged leaves and spider mite-infested leaves are depicted. For hop, the headspaces of clean leaves HCl-treated leaves and JA-treated leaves are depicted. The mean relative amounts are presented with the standard error (± SE). The mean total amount (± SE) of plant volatiles in area counts as measured by gas chromatography with the use of an FID is depicted in the figures as well. Bars for the same compound that are labeled with the same letter are not statistically significant (Kruskal Wallis test, alpha=0.05).

The compounds that are depicted in the figures are:

Aldehydes: **1.** (*Z*)-2-pentenal **2.** (*E,E*) or (*E,Z*) 2,4-hexadienal **3.** (*E*)-2-hexenal **4.** (*Z*)-2-hexenal **5.** (*Z*)-3-hexenal **6.** hexanal **7.** (*E*)-4-oxo-2-hexenal *Nitrogen containing compounds*: **8.** 2-methylbutanenitrile **9.** 3-methylbutanenitrile **10.** 2-methylpropanal, oxime **11.** 2-methylpropanal, O-methyloxime **12.** (syn) or (anti)-2-methylbutanal, oxime **13.** (anti) or (syn)-2-methylbutanal, oxime **14.** (syn) or (anti)-3-methylbutanal, oxime **15.** (anti) or (syn)-3-methylbutanal, oxime **16.** 2-methylbutanal, O-methyloxime **17.** 3-methylbutanal, O-methyloxime **18.** benzeneacetonitrile **19.** indole **20.** phenylacetaldehyde, O-methyloxime (tentative) *Ketones*: **21.** 2-butanone **22.** 1-penten-3-one **23.** 3-pentanone **24.** 4-methyl-3-penten-2-one **25.** 4-hydroxy-4-methyl-2-pentanone **26.** 1-octen-3-one **27.** (*Z*)-jasmone *Alcohols – non-terpenoid*: **28.** 2-methyl-1-propanol **29.** 1-penten-3-ol **30.** 2-penten-1-ol **31.** 2-methyl-1-butanol **32.** 3-methyl-1-butanol **33.** 1-pentanol **34.** 3-pentanol **35.** (*E*)-2-hexen-1-ol **36.** (*Z*)-3-hexen-1-ol **37.** 1-hexanol **38.** 2,4-pentanediol, 2-methyl **39.** 2-phenylethanol **40.** 1-octen-3-ol **41.** 3-ethyl-4-methylpentanol **42.** eugenol *Alcohols – terpenoid*: **43.** linalool *Carboxylic acids*: **44.** hexanoic acid *Esters*: **45.** 2-methylbutanoic acid, methylester **46.** (E)-2-hexenoic acid, methylester **47.** methyl benzoate **48.** 3-cyclohexen-1-ol, acetate **49.** (*E*)-2-hexen-1-ol, acetate **50.** (*Z*)-3-hexen-1-ol, acetate **51.** hexyl acetate **52.** methyl salicylate **53.** (*E*)-2-hexen-1-ol, butanoate **54.** (*Z*)-3-hexen-1-ol, butanoate **55.** hexyl butanoate **56.** (*Z*)-3-hexen-1-ol, tiglate **57.** (*Z*)-3-hexen-1-ol, 2-methylbutanoate **58.** (*Z*)-3-hexen-1-ol, hexanoate *Hydrocarbons - non-terpenoid*: **59.** 2-methylheptane (tent.) *Hydrocarbons – terpenoid*: **60.** limonene **61.** (*E*)-β-ocimene **62.** α-pinene **63.** (3*E*)-4,8-dimethyl-1,3,7-nonatriene **64.** (3*Z*)-4,8-dimethyl-1,3,7-nonatriene **65.** α-bergamotene **66.** β-caryophyllene **67.** α-copaene **68.** (*E*)-β-elemene **69.** (*Z*)-β-elemene **70.** (*Z,E*) or (*E,Z*)-α-farnesene **71.** (*E,E*)-α-farnesene **72.** (*E*)-β-farnesene **73.** germacrene A **74.** germacrene D **75.** α-humulene **76.** γ-muurolene **77.** α-selinene **78.** β-selinene **79.** γ-selinene **80.** (3*E*,7*Z*) or (3*Z*,7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene **81.** (3*E*,7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene *Ethers*: **82.** caryophyllene oxide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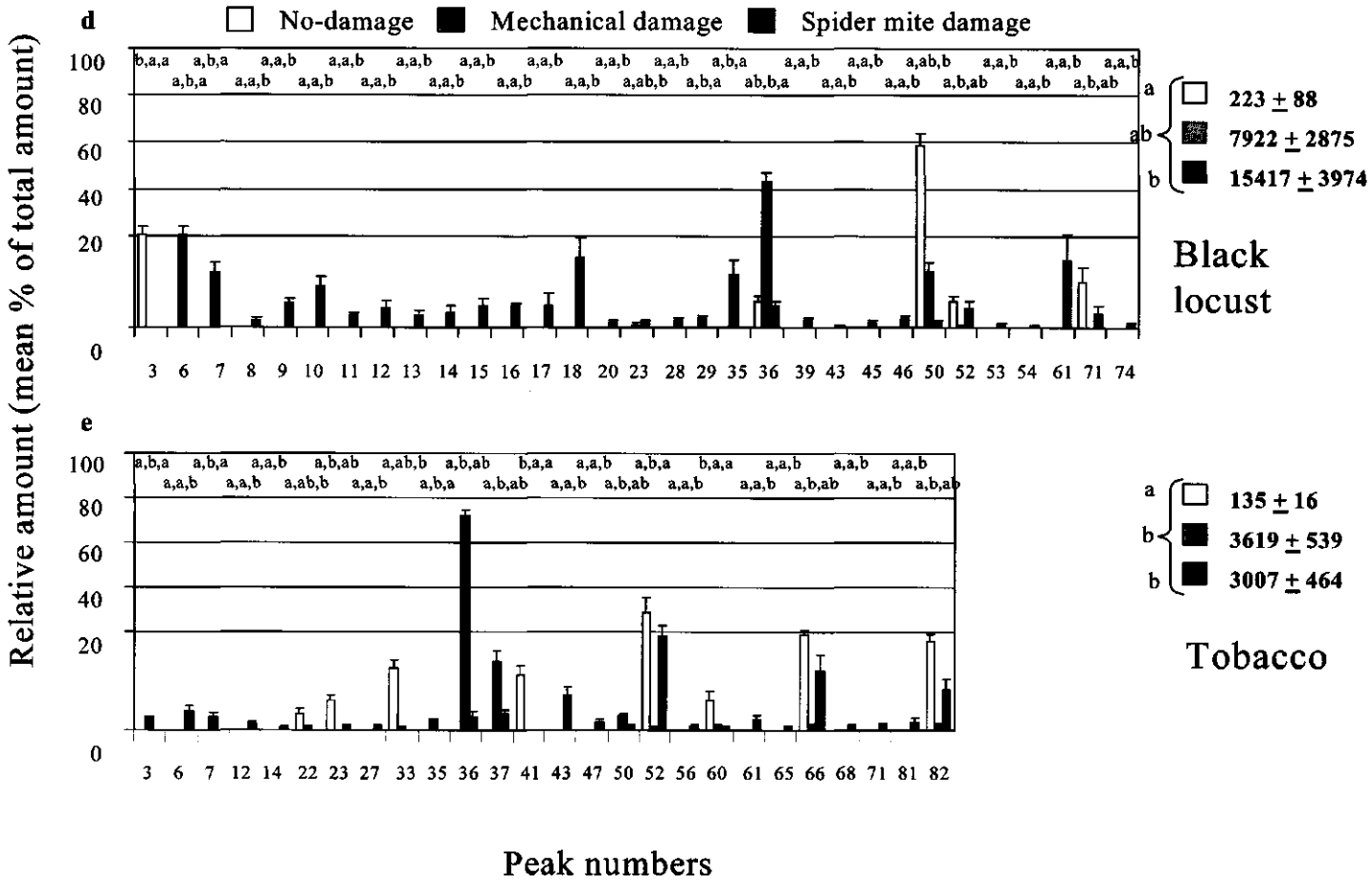
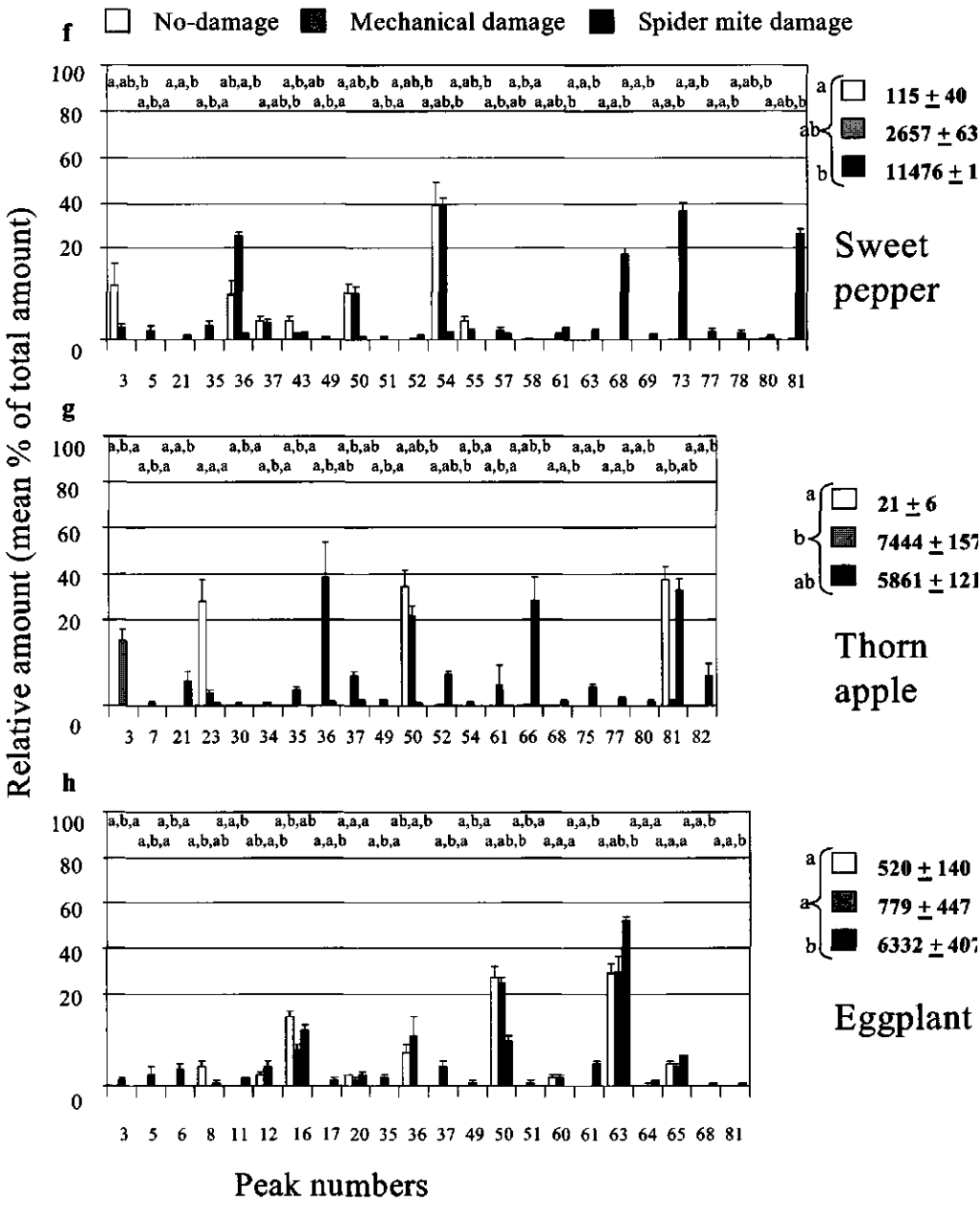
Mechanical damage
Spider mite damage
a
b
c
Relative amount (mean % of total amount)
Peak numbers
Golden chain
a 415 ± 103
ab 748 ± 127
b 8211 ± 2194
Soybean
a 1813 ± 719
ab 3975 ± 1072
b 18687 ± 5252
Cowpea
a 113 ± 33
ab 2191 ± 472
b 3588 ± 1327


d
No-damage
Mechanical damage
Spider mite damage
Relative amount (mean % of total amount)
100
80
60
40
20
0
3 6 7 8 9 10 11 12 13 14 15 16 17 18 20 23 28 29 35 36 39 43 45 46 50 52 53 54 61 71 74
a
ab
b
223 ± 88
7922 ± 2875
15417 ± 3974
Black locust
e
100
80
60
40
20
0
3 6 7 12 14 22 23 27 33 35 36 37 41 43 47 50 52 56 60 61 65 66 68 71 81 82
a
b
b
135 ± 16
3619 ± 539
3007 ± 464
Tobacco
Peak numbers


f
No-damage
Mechanical damage
Spider mite damage
Relative amount (mean % of total amount)
100
80
60
40
20
0
a,ab,b a,a,b ab,a,b a,b,ab a,ab,b a,ab,b a,ab,b a,b,a a,a,b a,a,b a,a,b a,ab,b
a,b,a a,b,a a,ab,b a,b,a a,b,a a,ab,b a,b,ab a,ab,b a,a,b a,a,b a,a,b a,ab,b
3 5 21 35 36 37 43 49 50 51 52 54 55 57 58 61 63 68 69 73 77 78 80 81
a
ab
b
115 ± 40
2657 ± 63
11476 ± 1
Sweet pepper
g
a,b,a a,a,b a,b,a a,b,a a,b,ab a,ab,b a,b,a a,ab,b a,a,b a,a,b a,a,b
a,b,a a,a,a a,b,a a,b,ab a,b,a a,ab,b a,b,a a,a,b a,a,b a,b,ab
3 7 21 23 30 34 35 36 37 49 50 52 54 61 66 68 75 77 80 81 82
a
b
ab
21 ± 6
7444 ± 157
5861 ± 121
Thorn apple
h
a,b,a a,b,a a,a,b a,b,ab a,a,a ab,a,b a,b,a a,b,a a,a,b a,a,a a,a,b
a,b,a a,b,ab ab,a,b a,a,b a,b,a a,b,a a,ab,b a,a,a a,ab,b a,a,a a,a,b
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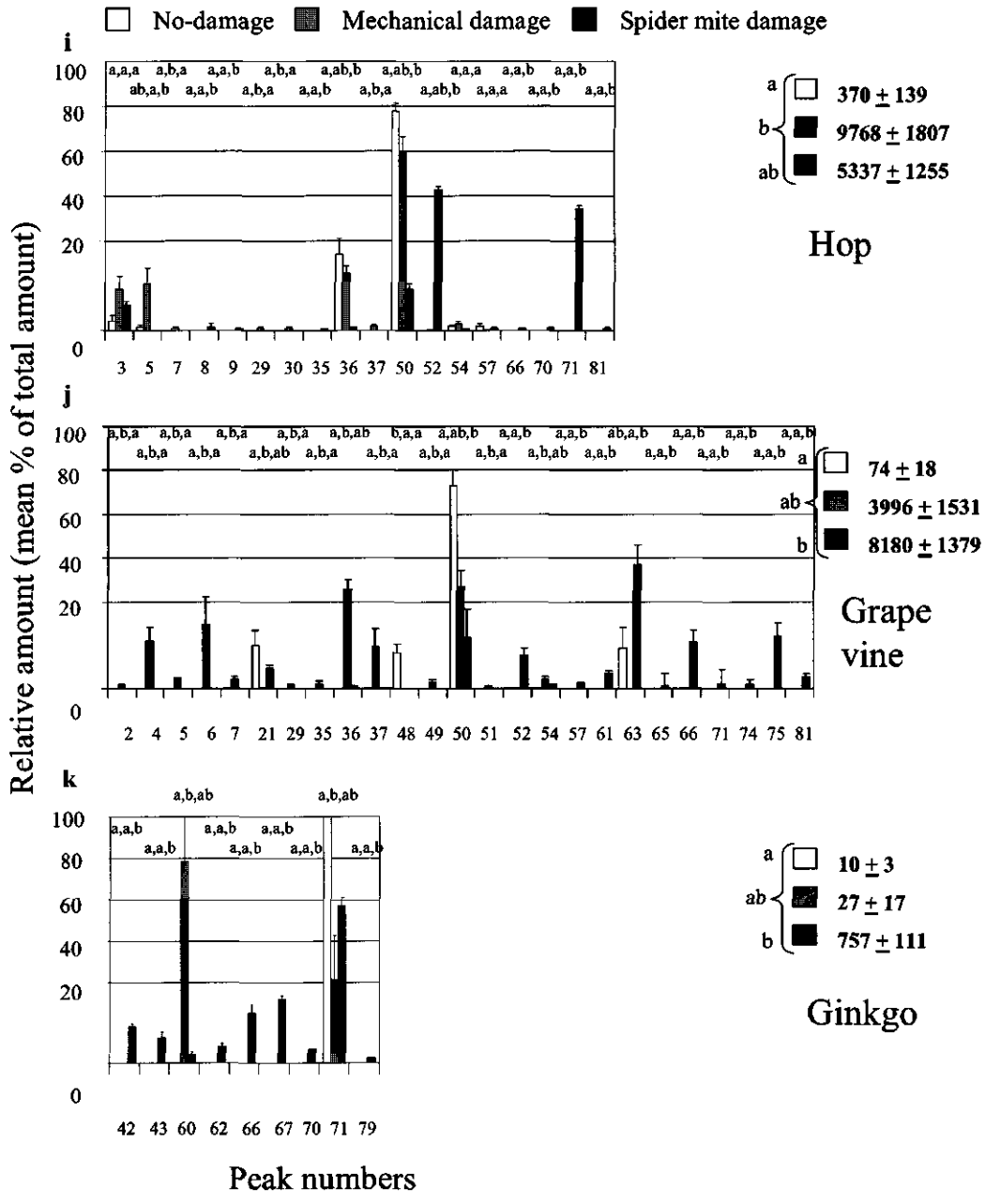
b
520 ± 140
779 ± 447
6332 ± 407
Eggplant
Peak numbers


No-damage
Mechanical damage
Spider mite damage
i
j
k
Relative amount (mean % of total amount)
Peak numbers
a 370 ± 139
b 9768 ± 1807
ab 5337 ± 1255
Hop
a 74 ± 18
ab 3996 ± 1531
b 8180 ± 1379
Grape vine
a 10 ± 3
ab 27 ± 17
b 757 ± 111
Ginkgo

Discussion

Production of synomones

Dicke et al. (1990a) reported that *T. urticae*-infested lima bean leaves were attractive to the predatory mite *P. persimilis*. They showed that the headspace of spider mite-infested lima bean leaves contained five major novel compounds. In 1999 when the sensitivity of the analytical equipment had considerably improved, Dicke et al. showed that there were many more novel compounds emitted from spider mite-infested lima bean leaves, however many of them were present in small amounts. The synomones linalool, methyl salicylate, (*E*)-β-ocimene and (3*E*)-4,8-dimethyl-1,3,7-nonatriene were significantly attractive to *P. persimilis* when individually offered to these predatory mites in an olfactometer (Dicke et al., 1990a). One or more of the synomone compounds that have been reported by Dicke et al. (1990a) were also induced in the spider mite-infested test plant species investigated in the present study, except for golden chain.

Methyl salicylate

Methyl salicylate was present in *T. urticae*-infested soybean, cowpea, tobacco and hop, as novel compound or as dominant compound in the blend. In black locust, sweet pepper, thorn apple and grapevine the compound was also present, albeit in lower concentrations. In the literature, the presence of methyl salicylate has been shown in the blend emitted by spider mite-infested leaves of apple, tomato, gerbera and especially lima bean plants (Dicke et al., 1998; Krips et al., 1999; Takabayashi et al., 1991). In contrast to the emission of methyl salicylate from spider mite-infested lima bean leaves, Ozawa et al. (2000) did not record methyl salicylate in the volatile blend emitted by lima bean leaves infested with caterpillars of *Spodoptera exigua* or *Mythimna separata*. Methyl salicylate was also not recorded from maize and cowpea plants treated with regurgitant of the

caterpillar *S. littoralis* (Fritzsche-Hoballah et al., 2002). This might raise the question whether methyl salicylate that is emitted by different plant species is specifically induced by the spider mite *T. urticae*. However, *Arabidopsis thaliana* infested with *Pieris rapae* caterpillars emitted methyl salicylate as major compound in the blend (van Poecke et al., 2001). Moreover, tobacco (*Nicotiana attenuata*) leaves infested with hornworm larvae (*Manduca quinquemaculata*) emitted a significantly larger amount of methyl salicylate than mechanically damaged leaves (Kessler and Baldwin, 2001). Shulaev et al. (1997) suggested that methyl salicylate is a volatile compound derived from salicylic acid, which is a key compound in the induced resistance in response to fungal, bacterial or viral pathogen attack (Metraux et al., 1990; Malamy et al., 1996; Ryals et al., 1995; Karban and Baldwin, 1997). Salicylic acid has also been reported to mediate whitefly-induced plant responses (Walling, 2000).

Nitrogen-containing compounds

In some plant species nitrogen-containing compounds such as oximes and nitriles are induced by the spider mites. The oximes were found in the headspace of golden chain, black locust, and eggplant. They have also been found in spider mite-infested lima bean, gerbera and cucumber and jasmonic acid-treated lima bean plants (Takabayashi et al., 1994b, Krips et al., 1999; Dicke et al., 1999). The headspace of spider mite-infested black locust plants contained, besides oximes, also the nitrogen containing compound 2- and 3-methylbutanenitrile. Kaiser (1993) suggested that these nitrogen-containing volatile compounds were produced from amino acids.

(E,E)-α-farnesene

Spider mite-infested golden chain leaves emitted the induced compound (*E,E*)-α-farnesene in large amounts. The compound was also induced in spider mite-infested soybean, black locust, tobacco, hop, grapevine and ginkgo. Moreover,

(*E*,*E*)-α-farnesene has been found in the headspace of *Psylla*-infested pear, together with methyl salicylate (Scutareanu et al., 1997). Both compounds attracted anthocorid predators in an olfactometer. Because (*E*,*E*)-α-farnesene is present in large amounts in the volatile blend of golden chain it might be a well detectable compound for the predatory mites. However, for golden chain it remains to be investigated whether spider-mite infestation results in attraction of predatory mites.

Qualitative versus quantitative differences

In response to spider mite-infestation each of the investigated plant species had its own volatile profile that comprised novel compounds (specific) but also compounds that were emitted in larger amounts compared to mechanically damaged leaves (non-specific). Dicke et al. (1998) considered a herbivore-induced blend to show qualitative differences compared to a blend from mechanically damaged leaves when the novel compounds are major blend contributors that are not produced in response to mechanical damage. The production of dominant novel compounds during spider mite-infestation has been shown for all tested plant species, except for eggplant and tobacco (both Solanaceae species). The dominant compounds in the volatile blend of spider mite-infested eggplant leaves consisted of non-specific compounds. Major compounds of the spider mite-induced blend of tobacco leaves resemble the major compounds of the blend emitted by clean leaves in the blend, but not those of mechanically damaged leaves, which is dominated by one green leaf volatile, i.e. (*Z*)-3-hexen-1-ol. Previous studies have shown that plant species of the Fabaceae (Dicke et al., 1990a; Du et al., 1998) show qualitative differences in their volatile blends when infested by spider mites, while plant species of the Solanaceae show mainly quantitative differences (Bolter et al., 1997; Dicke et al., 1998). The present study confirms this.

Direct versus indirect plant defense

The question whether plant species use a combination of direct and indirect plant defense has not been clearly answered yet. Dicke et al. (1998) hypothesized that plant species that show a quantitative difference between volatile blends emitted from herbivore-damaged and mechanically damaged plants have already a high level of direct defense. Therefore, these plant species have no ecological need to induce novel compounds to be more reliable for predators. To get an indication about the degree of direct defense of the investigated plant species, the degree to which the spider mite *T. urticae* accepted them has been investigated (van den Boom et al., 2003). The degree of indirect defense was indicated by the degree of predatory mite attraction towards plant odors of spider mite-infested plant species (van den Boom et al., 2002). Table 2 gives an overview of the direct and indirect defenses of all the investigated plant species.

Based on these results, it can be concluded that plant species with a low direct defense level use indirect defense to defend themselves, but do not always invest in the production of novel compounds. However, plant species with a high level of direct defense seem to invest in the production of novel compounds as well. For example, for a ginkgo tree it does not seem necessary to invest in specific compounds to strengthen the use of indirect defense while this plant already possesses a very strong direct defense. However, ginkgo leaves showed the emission of novel compounds that dominated the blend after treatment with jasmonic acid. This may indicate that the ability of plants to induce bioynthetic pathways that result in the emission of novel volatiles has originated very early in the evolution of plants.

Table 2. Direct versus indirect defence

Plant species	**Direct defence** [1]	**Indirect defence** [2]	**Specificity of indirect defence** [3]
Fabaceae			
Soybean	Very weak	Very strong	High
Golden chain	Very weak	- [4]	High
Black locust	Weak	-	High
Cowpea	Weak	Less strong	High
Solanaceae			
Tobacco SR1	Very weak	Less strong [5]	Low
Eggplant	Weak	Less strong	Low
Thorn apple	Less strong	-	High
Sweet pepper	Strong	Very strong	High
Other families			
Hop	Very weak	Less strong	High
Grapevine	Less strong	Very strong	High
Ginkgo	Very strong	Less strong	High

[1] Direct defence in percentages of *T. urticae* that had left a leaf disc of the indicated plant species within 15 minutes (van den Boom et al., 2002a): Very strong: 80-100 %, Strong: 60-80 %, Less strong: 40-60 %, Weak: 20-40 %, Very weak: 0-20 %.

[2] Indirect defence in significant percentages when at least forty predators of *P. persimilis* were tested on infested leaves versus uninfested leaves in an olfactometer (van den Boom et al., 2002b). For all plant species the predatory mites were significantly attracted towards the infested leaves, but the significance in attraction varied from very strong to less strong. Very strong: significance of $P \leq 0.001$, Less strong: $0.05 \geq P > 0.01$.

[3] Specificity of indirect defence: High: presence of novel compounds that are dominant in the blend, Low: presence of novel compounds that are only present in small amounts.

[4] Not investigated

[5] M. Dicke and H. Dijkman. (unpublished results)

Conclusion

All the investigated plant species emitted one or more novel compounds when spider mite-infested leaves were compared to mechanically damaged leaves. This shows that during spider-mite feeding on different plant species several new biosynthetic pathways were induced. The induction of novel pathways and compounds is a sophisticated way of indirect defense, because it is likely to increase the probability that the predatory mites discriminate herbivore-infested plants from mechanically damaged plants. It was hypothesized that plant species with a low degree of direct defense would invest in indirect defense by the induction of specific volatiles. Indeed, qualitative differences in spider mite-induced volatile blends are more prominent in the Fabaceae than in the Solanaceae. However, this trade-off is not obvious for plant species with a low or very low direct defense level against spider mites, such as tobacco and eggplant. They do not invest in the production of novel compounds. For tobacco however, it is known that the plant invests in direct defense by the production of nicotine in response to herbivore attack. This induced response has considerable costs in terms of fitness (Baldwin, 1999). For all investigated plant species, minor induced specific compounds or synergism between compounds might be responsible for predatory mite attraction. Therefore, more research must be carried out to show which compounds are important in the tritrophic interaction of each of these plant species, the spider mite *T. urticae* and the predatory mite *P. persimilis*.

Acknowledgements

I thank Henk Smid and Pieter Kuiken of Unifarm Wageningen for the cultivation of the investigated plant species, Herman Dijkman for the spider mite-rearing on the lima bean plants, and Elbert van der Klift with the maintenance of the gas chromatograph.

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Development of a gas chromatographic fractionation method to manipulate volatile blends in order to identify bioactive volatiles

Abstract

Volatile infochemicals are important mediators of interactions within and among species. Often, the active components mediating these interactions are part of a complex mixture, which makes their chemical and biological identification difficult. Besides, the profile of the investigated volatile fractions after processing can differ from the chemical profile of the original blend. The aim of the new method developed here was to get a selective and more efficient way for the biological identification of bioactive compounds compared to conventional methods. Therefore, a fractionation method was developed in which separation of the volatile mixture on a gas chromatographic column was used. One or more components could selectively be removed from the mixture. The remaining components (or the intact mixture), which had similar ratios compared to the original blend, were trapped onto an adsorbent. With thermal desorption the components were revolatilized and stored in a Teflon bag before they were tested in a bioassay. An olfactometer bioassay was used to test the bioactivity of the fractionated blend. With this new method solvent introduction into the bioassay is circumvented. The method was validated for a broad range of volatiles with different boiling points that belong to groups of different chemical nature. Most of the investigated compounds showed 80-100 % recovery. However, compounds with a relatively high boiling point (> 300 °C) or with a phenolic group showed only 30-50 % recovery. To validate the method on bioactivity, headspace of *Tetranychus urticae*-infested lima bean leaves was offered to the predatory mite *Phytoseiulus persimilis* after processing to assess whether the predators reacted to the induced volatiles in the olfactometer. Besides, the

compounds methyl salicylate and (3*E*)-4,8-dimethyl-1,3,7-nonatriene which were known to attract predatory mites were tested in the olfactometer after processing.

Introduction

Infochemicals play an important role in plant-insect and insect-insect interactions. A lot of studies have been carried out to reveal the molecular structures of active compounds. On-line or off-line trapping techniques have been used to adsorb the volatiles onto an adsorbent material (Bicchi and Joulain, 1990; Raguso and Pellmyr, 1998; Röse et al., 1996). Subsequently, the volatile blend has been recovered by chemical or thermal desorption.

A drawback of chemical desorption is that a solvent is needed to desorb the compounds. As a result, the extract is diluted, which means that often it will be necessary to concentrate the solution again. Paraffin oil can also be used to dissolve the volatiles before concentration of the solution takes place, to minimise the loss of the compounds of interest during evaporation of the solvent. However, introduction of a solvent into the bioassay may disturb the insect's behaviour, because the solvent can obscure the volatile bioactive compounds to be investigated. Also, impurities in the solvent or paraffin oil can contribute to the disturbance of insect behaviour. In addition, the most volatile compounds will be overrepresented in the volatile blend presented to the insect.

Thermal desorption has some disadvantages as well. It is well known that thermal breakdown of adsorbed compounds or unstable adsorbent material may take place above certain temperatures, which can lead to the production of artefacts in the volatile mixture (Agelopoulos and Pickett, 1998). An advantage of thermal desorption is that this technique can circumvent the need of solvent

introduction into the bioassay because the volatiles are recovered in the vapour state.

To investigate if a volatile mixture is biologically active, bioassays can be used such as windtunnels (Bolter et al., 1997; Du et al., 1996; Geervliet et al., 1996; Powell et al., 1997; Turlings et al., 1991a,b; van Poecke et al., 2001) or olfactometers (Dicke et al., 1988; Takabayashi et al., 1998). In these bioassays fractions or compound mixtures dissolved in a solvent or in a vapour phase can be tested. A drawback is that due to the non-linear evaporation rate of the solvent the profile of the manipulated blend can differ from the chemical profile of the original blend. Besides, solvent introduction into the bioassay can disturb the insect's response.

The active compounds in a volatile blend that mediates an interaction within or between insect species are often part of a complex mixture. For chemical identification, gas chromatography alone (GC) or in combination with mass spectrometry (GC-MS) can be used to analyze the volatiles. However, biological identification of compounds that contribute to the bioactivity of a complex blend is more difficult. For example, the volatile blend of spider mite-infested gerbera consists of more than one hundred compounds (Krips et al., 2001). There are different techniques to identify the bioactive compounds in a volatile blend, although these conventional techniques have encountered some difficulties in the past. One of these methods that should be considered is the comparison of volatile profiles. For instance, the volatile blend produced by a male insect can be compared to the volatile blend produced by a female insect. However, this technique becomes complicated when the blend is a complex mixture.

Another method that can be used for biological identification, is the fractionation of a volatile blend to isolate and test compounds or fractions that stimulate insect

attraction. When a fraction is biologically active, combinations of active fractions can be investigated to see if they lead to stronger insect attraction (additive effects). When the combination of fractions leads to insect attraction that is stronger than the sum of the individual fractions, synergism occurs. Another possibility is to use subtractive fractionation. With this method one or more compounds are eliminated from the complete volatile mixture. With subtractive fractionation fewer trials are needed to test all possible combinations (Byers, 1992). In this way the possibility of synergism and additive effects can be revealed more easily. Subtractive-fractionation methods have been less well studied than additive-fractionation methods.

Another way to show bioactivity can be done by testing synthetic compounds. However, it is hard to mimic an original volatile blend with a synthetic mixture, because minor compounds that are difficult or impossible to detect by analytical methods can be important for the biological activity of the blend. Moreover, the synthetic compounds are not always available and also the mixing of these compounds in the 'natural' ratios can be difficult. For example, the parasitoid *Cotesia marginiventris* was less attracted to a synthetic blend of eleven compounds than to the natural blend of volatiles released from corn seedlings on which beet armyworm larvae were feeding (Turlings et al. 1991b). However, preflight experience with the synthetic blend improved the responses of the wasps. When individual synthetic compounds of the blend of Red Delicious apples were tested on their attractiveness towards the apple maggot flies *Rhagoletis pomonella*, they did not show a similar attractiveness compared to the natural blend. Only a mixture of synthetic compounds in the right ratios attracted the apple maggot flies *Rhagoletis pomonella* to the same extent as the natural blend when tested in a bioassay (Fein et al., 1982).

When the suitability of these methods for biological identification is compared, fractionation of volatile mixtures seems the most selective way to identify bioactive compounds in complex mixtures. Therefore, a new method based on fractionation is presented and validated in this chapter. In this method chromatographic separation was used to remove the non-active fractions from the mixture. In this way the volatile profile of the manipulated blend still highly resembles the original blend minus the subtracted fractions. After chromatographic separation, the volatiles were trapped onto an adsorbent and thermal desorption was used to bring the compounds in the volatile phase again, to circumvent solvent introduction into the bioassay. A fraction of the manipulated blend or the intact volatile blend was stored in a Teflon bag and subsequently tested in an olfactometer. The tritrophic system of the spider mite *Tetranychus urticae*, the predatory mite *Phytoseiulus persimilis* and lima bean plants was used to validate the method, because both behavioural and chemical identification of a number of compounds in this interaction had already been identified by Dicke et al. (1990).

Materials and methods

Chemical components

Standard solutions of several compounds were prepared at a concentration of 100 ppm. tert-Butyl methyl ether (TBME) 99 % obtained from Acros Organics was used as solvent. For the preparation of the solutions the following synthetic compounds were used; 5-hexen-2-one (unknown origin), (*Z*)-3-hexen-1-ol, acetate 98 % (Fluka), (*E*)-β-ocimene (unknown origin), (*E,E*)-farnesol 96 % (Merck), (*E*)-nerolidol 98 % (Fluka), (*Z*)-3-hexen-1-ol 98 % (Fluka), (-)-β-pinene 98% (Jansen Chimica), methyl salicylate 99 % (Acros Organics) and β-caryophyllene >98.5 % (Roth). The compound (3*E*)-4,8-dimethyl-1,3,7-nonatriene was synthesised in our laboratory and had a purity of 90 % (Dicke et al., 1990). Figure 1 presents the formulas of all investigated compounds.

Plant material

Lima bean plants (*Phaseolus lunatus* L.) were grown in the greenhouse facility of Wageningen University at a temperature of 20 ± 2 °C, a relative humidity of 60-80 % and a photoperiod of L16:D8 hours. Spider mite-infested lima bean plants were kept in a separate greenhouse. The conditions of this greenhouse were 20-30 °C, r.h. of 60-80 % and L16:D8.

Mite rearing

The spider mites, *Tetranychus urticae* Koch (Acari: Tetranychidae), were reared on lima bean plants. Predatory mites, *Phytoseiulus persimilis* A.-H., were originally obtained from Entocare cv, Wageningen, The Netherlands, and reared on spider mite-infested lima bean leaves in Petri dishes sealed with parafilm. The Petri dishes were stored in a temperature controlled room at 23 ± 2 °C, r.h. 60-80 % and 16L:8D. Every two or three days fresh spider-mite infested leaves were

added to the dishes. For the olfactometer experiments only young adult females of *P. persimilis* were used, whose age was 1-7 days since the final moult.

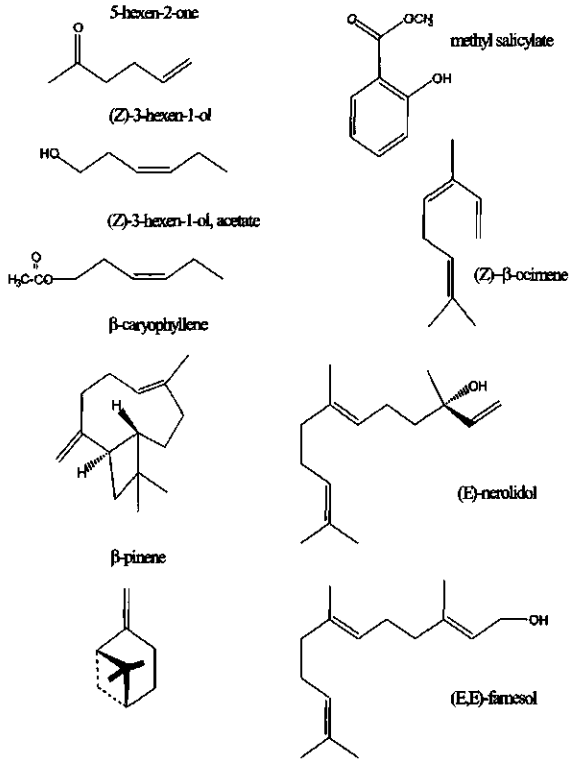

Figure 1: Formulas of the compounds used in the test solution.

Headspace setup: volatile trapping

The parallel headspace setup with six jars described in Chapter 4 was used to trap plant volatiles on Tenax adsorbent in glass tubes (Gerstel: 160 mm long * 6.0 mm OD, 4 mm ID). For the volatile collection spider mite-infested lima bean leaves were detached after three days of infestation and five trifoliate leaves were placed with their petioles in wet cotton wool in each glass jar. Volatiles were collected for 2 hours and were used to test the attraction of predatory mites to the

components in the olfactometer setup after they have passed through the fractionation system.

Fractionation chromatography

A two-dimensional gas chromatograph (HP 5890) connected to the fractionation system was used to separate the volatiles and recollect them on a fractionation tube filled with Tenax (Figure 2).

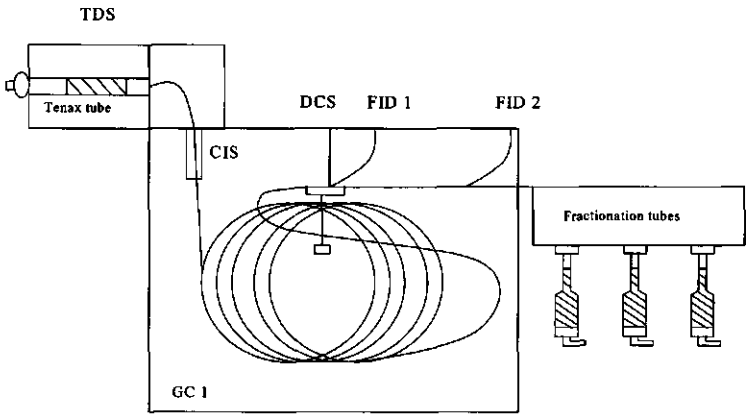

Figure 2: Gas chromatograph with Thermal Desorption System (TDS) connected to a fractionation system with tubes filled with Tenax to recollect the compounds. A Dual Column Switching device (DCS) can be used eliminate certain compounds from the volatile blend. CIS = Cold Injection System, FID = Flame Ionisation Detector.

The second oven of the gas chromatograph was only used to transfer the volatiles to the fractionation system. Besides manual injection of the solution of standard compounds, a TDS-system was used as well to transfer the solution of standard compounds with a hydrogen flow into the gas chromatograph. When the TDS-system was used, the compounds were trapped again in the liner (1.4 mm ID) of the injector of the gas chromatograph that was cooled with CO_2 to –70 °C. An identical temperature program as in Chapter 4 was used for the TDS.

After separation of the compounds in the GC column, selected compounds could be removed from the whole mixture with a Dual Column Switching device (DCS, Gerstel, Germany). A countercurrent gas flow greater than the hydrogen carrier gas flow was used to remove the selected compound from the carrier gas stream (Figure 3).

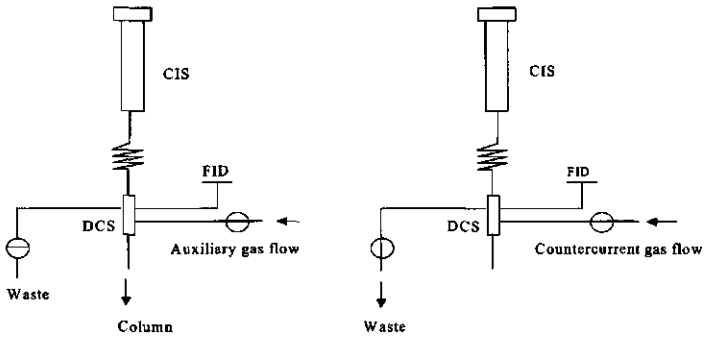

Figure 3: *Schematic drawing of the Dual Column Switching device (DCS).*
Left: When the valve towards the waste is closed and a low auxiliary gas flow is present, compounds continue their way to the fractionation system.
Right: When the valve towards the waste is open and the countercurrent gas flow is higher than the column flow, compounds are transferred to the waste.

When the auxiliary gas flow was smaller than the carrier gas flow, the compounds continued their way to the fractionation system. The difference in auxiliary and countercurrent gas flow was regulated with a pressure controller. An FID was connected to the DCS to quantify the complete mixture of compounds going through the column. After the DCS, a second FID was installed to confirm that the right compound had been taken out of the mixture.

The non-selected compounds continued with the carrier gas and were recollected in the fractionation system on one separate fractionation tube filled with Tenax.

The gas chromatograph had an initial temperature of 40 °C. After 2 min the temperature of the oven was programmed at a rate of 4 °C/min to 220 °C. The standard solution was injected in splitless mode and the injector was ramped with 12 °C/sec from 150 °C to 250 °C. The transfer lines to the fractionation system and the splitter to the different Tenax tubes were kept at 200 °C as well. A megabore column (Chrompack, CP-SIL 8, 25 m long, 0.53 mm ID, 1.0 μm film thickness) was used to allow high column loads. As transfer line a deactivated capillary tube was used. The end of the transfer line was connected to a fractionation tube (broad part: 95 mm long, 7 mm OD, 6 mm ID; narrow part: 37 mm long, 2mm OD, 1 mm ID). The Tenax adsorbent was held in place with plugs of silanized glass wool. The narrow part of the tube was connected to the fractionation system by a Teflon lined nut of stainless steel. On top of the outer part a screw cap was placed, enclosing a glass connector. The hydrogen flow from the column was led through one of the Tenax tubes and via silicone tubing to an on/off flow valve.

Volatiles transfer (preparation of odour source)

After recollection of the volatiles in a fractionation tube filled with Tenax, the tube was placed in an oven that was specially designed for these tubes (Desaga, GmbH, Germany) (Figure 4).

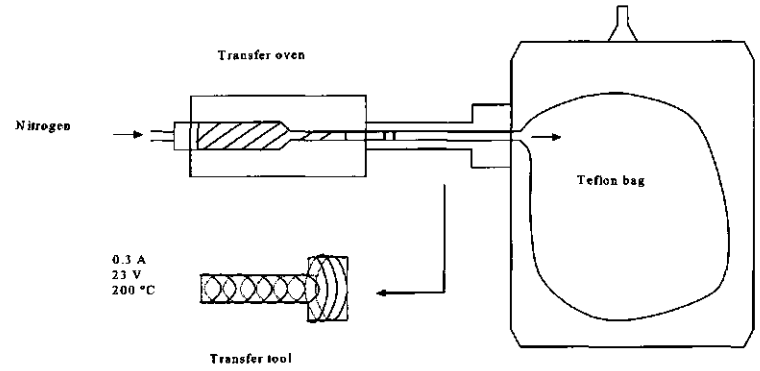

Figure 4: Schematic drawing of a transfer oven in which volatiles trapped on a fractionation tube can be desorbed; the tube is connected via a heated transfer tool (see close-up) to a Teflon bag placed in a jar. The transfer tool can be heated by applying a voltage over its windings.

On one side a nitrogen cylinder was connected to the fractionation tube, on the other side the fractionation tube was connected to a Teflon bag (3 L) placed in a bigger jar (20 L). The connections were made with Teflon tubing. Before use, the Teflon bag had been cleaned overnight in an oven by continuously flushing it with room air at a temperature of 110 °C. The connection between the narrrow glass part of the fractionation tube filled with Tenax and the bag was made from Teflon connectors and Teflon tubing. The Teflon tubing was surrounded by a heated transfer tool; narrow part: 40 mm long, 6 mm OD, 4 mm ID; broader part (screw): 12 mm long, 8 mm OD, 6 mm ID that could be heated to 200 °C. The transfer tool could be connected to the jar by screwing the device onto the jar. The aluminum surface of the tool had been made non-conductive by electrochemical oxidation. An electrical resistance wire was wrapped around it and the wire was connected to a power supply (NEP 613, 0-30 V, 2.5 A). The compounds were desorbed within 10 min from the Tenax tube by heating the oven and the transfer part to 200 °C (23 V, 7 W). The transfer part was heated to

reduce the risk of condensation of compounds with high boiling points in the transfer line. The nitrogen flow through the tube was about 50 ml/min.

Method validation

To check the suitability of the method for a range of volatiles, recoveries were measured of several reference compounds differing in boiling point and chemical nature. To obtain a reference value 5.0 µl of a mixture, which contained the compounds 5-hexen-2-one, (*Z*)-3-hexen-1-ol, acetate, (*E*)-β-ocimene, (3*E*)-4,8-dimethyl-1,3,7-nonatriene, (*Z*)-3-hexen-1-ol, β-pinene, methyl salicylate and β-caryophyllene, at a concentration of 100 ppm of each compound dissolved in TBME, was injected onto the Tenax material in a fractionation tube (Figure 5, experiment 0). After injection, the compounds were subsequently transferred to a headspace tube filled with Tenax via the specially shaped transfer oven and the heated transfer tool. This was replicated three times to obtain a mean reference value to calculate recoveries of all compounds. Four different experiments were carried out and three replicates were obtained for each experiment (see Figure 5 for a schematic drawing).

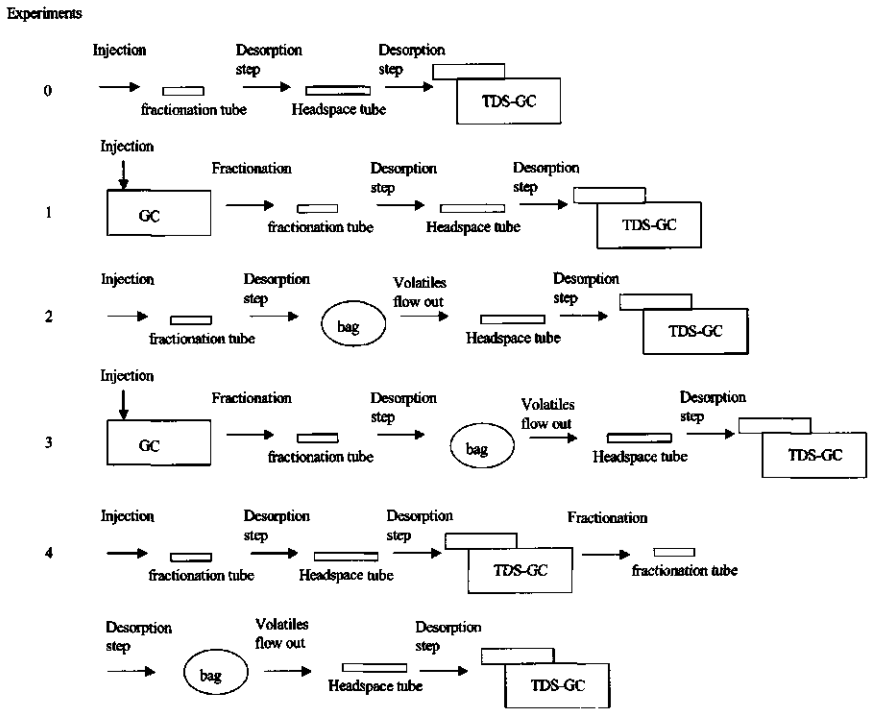

Figure 5: Schematic representation of experiment 0 (reference experiment) and experiments 1 to 4 (method validation). These experiments were carried out to show the recoveries of the reference compounds. A detailed description of the experiments is given in Materials and Methods.

To obtain the reference values, experiment 0 was more reliable than direct injection of the solvent into tubes filled with Tenax, because during the time that was necessary to bring the tubes into the TDS, compounds were lost due to solvent evaporation.

Experiment 1: 5 µl of the standard solution was manually injected into the gas chromatograph (Figure 2). After separation the volatiles were trapped onto Tenax in a fractionation tube. Subsequently, they were transferred with nitrogen to a headspace tube filled with Tenax via thermal desorption in an oven and a heated

transfer tool (see Figure 4). The headspace tube was desorbed in a TDS and the volatiles were analyzed by GC-FID.

Experiment 2: 5 µl of the standard solution was injected onto Tenax in a fractionation tube. The fractionation tube was placed into the oven. The components were desorbed at 200 °C and transferred with nitrogen via a heated connection tube to a Teflon bag in a jar (Figure 4). After 10 minutes the transfer was ended. The bag was disconnected from the heated transfer tool and the bag was connected to a headspace tube filled with Tenax. The volatiles were pressed out of the Teflon bag by blowing air into the jar on the outside of the Teflon bag, to displace the gas volume in the Teflon bag. The volatiles that were pressed out of the Teflon bag were collected on Tenax in a headspace tube. The headspace tube was desorbed in a TDS and the volatiles were analyzed with GC-FID.

Experiment 3: 5 µl of the standard solution was manually injected into the gas chromatograph (Figure 2). After separation the volatiles were adsorbed onto Tenax in a fractionation tube. Afterwards, the compounds were thermally desorbed and transferred with nitrogen via a heated connection tube to a Teflon bag in a jar (Figure 4). After 10 minutes the transfer was ended. The bag was disconnected from the heated transfer tool and the bag was connected to a headspace tube filled with Tenax. The volatiles were pressed out of the Teflon bag by blowing air into the jar on the outside of the Teflon bag, to displace the gas volume in the Teflon bag. The volatiles that were pressed out of the Teflon bag were collected on Tenax in a headspace tube. The headspace tube was desorbed in a TDS and the volatiles were analyzed with GC-FID.

Experiment 4: 5 µl of the reference solution was injected onto Tenax in a fractionation tube and the components in the tube were subsequently transferred via the heated oven and transfer tool to a headspace tube filled with Tenax. The tube was brought into a TDS-system and the components were transferred into the liner of the gas chromatograph. The other steps were identical to the steps described in experiment 3. The volatiles were analyzed by GC-FID.

More reference values were obtained with another standard mixture dissolved in TBME (Figure 5, experiment 0), which contained two compounds with a higher molecular weight and boiling point: (*E,E*)-farnesol and (*E*)-nerolidol. For this standard solution only the first (Figure 5, experiment 1) and the fourth experiment (Figure 5, experiment 4) described above were carried out. Of these experiments, also three replicates were obtained.

Connection of odour source to olfactometer

After the Teflon bag had been filled with volatile components, the bag was connected to an olfactometer setup as described by Takabayashi and Dicke (1992) (Figure 6). The volatiles were pressed out of the bag by blowing air into the jar between the Teflon bag and the jar's wall, displacing the mixture in the bag. The continuous flow of volatiles that came out of the bag was around 50 ml/min for about one hour. Make-up air was used to transfer the volatiles to the olfactometer. The airflow in each arm of the olfactometer was 4 l/min and vacuum was applied on the downwind side of the olfactometer where the predatory mites were introduced into the olfactometer. Adult female predatory mites had been starved for 1-3 h before they were introduced at the downwind side of the olfactometer and were allowed to walk upwind on an iron wire in the Y-tube. At the junction they could make a choice between the two odour flows coming from the two arms. When the predatory mite had reached the end of one of the arms her choice was recorded and the mite was discarded. However, when the end of one of the arms was not reached within 5 min, the predatory mite was removed and excluded from statistical analysis.

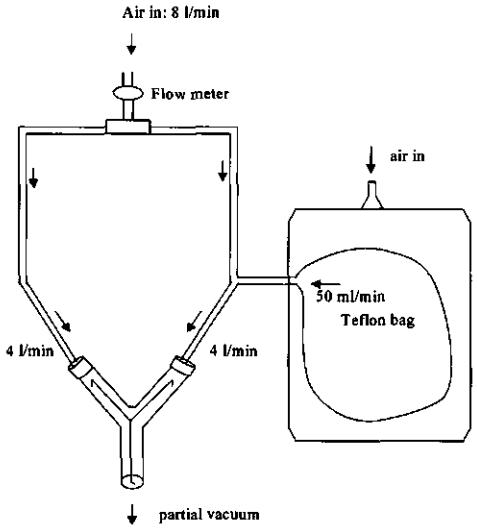

Figure 6: Flows leading towards the olfactometer. The volatiles in the Teflon bag are pressurised and displaced by air that is flowing into the jar. A flow of 50 ml/min containing the compounds is transferred to one arm of the olfactometer with a make-up air flow of 4 l/min. the other arm of the olfactometer is provided with an air flow of 4 l/min serving as control.

Method evaluation

Olfactometer experiments with plant volatiles were carried out to test if the volatile blend was still attractive to predatory mites after the thermal desorption steps. Methyl salicylate and (3*E*)-4,8-dimethyl-1,3,7-nonatriene were used to test the sensitivity of the predatory mites to these plant synomones after the fractionation method had been used to desorb and transfer these volatiles into the Teflon bag.

Experiment 1: Plant volatiles were trapped on the Tenax adsorbent material with the use of the parallel headspace collection system. Four replicate headspace tubes with the collected volatiles were individually desorbed in the TDS and the compounds were subsequently transferred into the gas chromatograph. After

separation, the compounds were collected on Tenax in a fractionation tube and thermally transferred to the Teflon bag. After storage of the compounds in the bag, they were used in an olfactometer experiment.

Experiment 2: A solution of 5 µl methyl salicylate / 10 ml TBME (500 ppm) was prepared and 5 µl of the solution were injected into the gas chromatograph (2.5 µg of methyl salicylate). After collection of methyl salicylate on a fractionation tube, the compound was thermally transferred to the Teflon bag. This procedure was repeated once more before the bag was used in the first olfactometer test (in total 10 µl solution; 5 µg methyl salicylate) and twice for the second olfactometer test (in total 15 µl solution; 7.5 µg methyl salicylate). After storage of the volatiles in the bag, the bag's contents were used in an olfactometer experiment.

Experiment 3: A solution of 10 µl (3*E*)-4,8-dimethyl-1,3,7-nonatriene / 10 ml TBME (1000 ppm) was prepared and 5 µl of the solution was injected into the gas chromatograph (5 µg of (3*E*)-4,8-dimethyl-1,3,7-nonatriene). After the collection of (3*E*)-4,8-dimethyl-1,3,7-nonatriene onto the fractionation tubes the compound was thermally transferred to the Teflon bag. This procedure was repeated once before the Teflon bag was used in the olfactometer test (in total 10 µl solution; 10 µg (3*E*)-4,8-dimethyl-1,3,7-nonatriene). After storage of the volatiles in the bag, they were used in an olfactometer experiment.

Statistics

Kruskal Wallis tests combined with multiple comparison tests were performed for the method validation to analyze differences among the recoveries in the different experimental steps for each component. One-sided binomial tests were performed on the number of predatory mites in each of the olfactometer arms. To evaluate if the responses of the predatory mites between two replicate experiments were significantly different, a 2x2 contingency table test was used.

Results

Method validation

The method was validated with a mixture of several reference compounds differing in boiling point and chemical nature. These compounds were quantitatively analyzed before and after the different steps in the method. This was primarily done to see if the method was suitable for a broad range of volatiles with different boiling points that belong to groups with a different chemical nature.

Recoveries of reference compounds

In the first experiment the standard solution was separated by gas chromatography and subsequently recollected onto a fractionation tube filled with Tenax (Figure 5, experiment 1). This tube was desorbed again in an oven and the compounds were recollected onto a headspace tube filled with Tenax. After these steps, recoveries of roughly 80-100 % were obtained (Figure 7). Only one compound, (*E*)-nerolidol, had a recovery of 60 %. In the second experiment the standard solution was brought onto Tenax in a fractionation tube and was subsequently desorbed in an oven. The compounds were transferred via the heated connection to the Teflon bag and after a while pressed out of the bag again and trapped onto a headspace tube filled with Tenax (Figure 5, experiment 2). The highest loss in recovery in these experimental steps was obtained for methyl salicylate. Recovery was reduced from 81 to 41 %, although no significant difference was found due to high standard deviations. The third experiment was a combination of the first and second experiment. After the gas chromatographic separation, the fractionation tube on which the compounds were collected was used for desorption of the compounds into the Teflon bag (Figure 5, experiment 3).

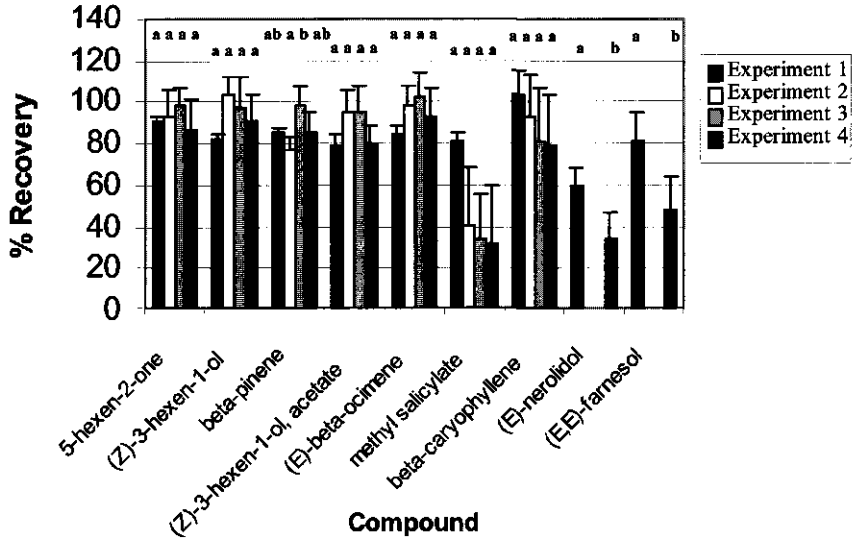

Figure 7: Recoveries of compounds (mean ± SD) used to evaluate the fractionation system in experiments 1 to 4 (for description of the 4 experiments see the text). For statistical analysis the Kruskal Wallis test was used. Bars for the same compound that are marked with the same letter are not significantly different. Bars that are marked with the letters 'a/b' are not significantly different to either 'a' or 'b'.

Only for α-pinene a significant difference between the second and third experiment was revealed ($P < 0.05$). The fourth experiment comprised the whole experimental process. In this case, the third experiment was repeated, but the standard solution injected onto Tenax in a fractionation tube was first transferred to Tenax in a headspace tube. This tube (normally obtained via headspace trapping of the plant volatiles) could be brought into the gas chromatograph via a TDS-system (Figure 5, experiment 4). No significant differences between the recoveries of the overall experiment were obtained when compared to the other experiments for any compound except (*E*)-nerolidol and (*E,E*)-farnesol (Figure 7). For the latter two compounds only the first and the fourth experiment were conducted. For these compounds the recoveries of the fourth experiment were

significantly reduced compared to the first experiment. To show that it was possible to take one compound out of a mixture, chromatrograms were recorded of the test mixture before the DCS (FID 1) and after the DCS (FID 2) (Figure 8). The compound methyl salicylate has selectively been removed from the test mixture.

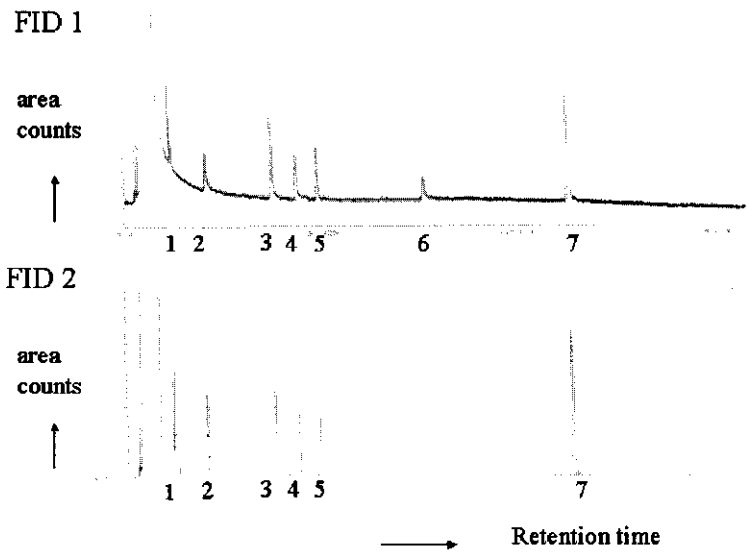

Figure 8: The chromatogram above shows the whole test mixture injected into the gas chromatograph measured by FID 1. The chromatogram below shows the mixture after it has passed the Dual Column Splitter. The compound methyl salicylate has been selectively taken out of the mixture, as shown by FID 2. The compounds shown in the chromatograms are: 1) 5-hexen-2-one 2) (*Z*)-3-hexen-1-ol, 3) β-pinene 4) (*Z*)-3-hexen-1-ol, acetate 5) (*E*)-β-ocimene 6) methyl salicylate 7) β-caryophyllene

Biological activity of headspace compounds after processing

The method was evaluated with a headspace collection of *Tetranychus urticae*-infested lima bean volatiles on Tenax in an on-line headspace system. The predatory mite *Phytoseiulus persimilis* was used in an olfactometer experiment to

assess whether they reacted upon the induced volatiles after the volatiles had undergone fractionation, thermal desorption and storage in a Teflon bag. The olfactometer experiment showed that predatory mites were strongly attracted to spider mite-induced volatiles from lima bean leaves after the fractionation and thermal desorption steps (Figure 9). The attraction of the predatory mites towards the plant volatiles was highly significant ($P = 1.1x10^{-5}$).

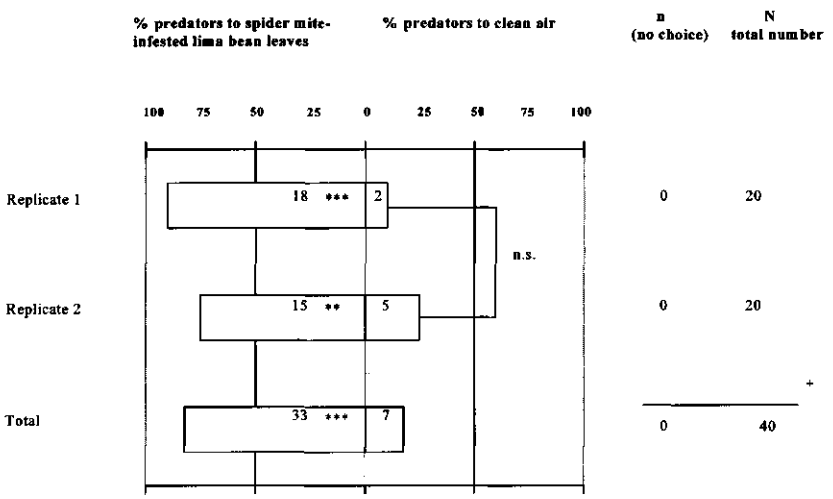

Figure 9: Spider mite infested lima bean volatiles were tested versus clean air in an olfactometer in two replicate experiments. The volatiles have been trapped and transferred through the fractionation system via thermal desorption into the Teflon bag. The response of adult females of the predatory mite *P. persimilis* is depicted. Statistics: one-sided binomial test and a 2x2 contingency table test (*** = $P \leq 0.001$, ** = $0.01 \geq P > 0.001$).

Biological activity of individual compounds after processing

To show the sensitivity of the method, low concentrations of the individual compounds methyl salicylate and (3*E*)-4,8-dimethyl-1,3,7-nonatriene were tested. These two compounds are known to be induced by *T. urticae* feeding on certain host plants (see Chapter 4, this thesis / 'van den Boom et al., in prep.')

and are attractive to *P. persimilis* in a certain concentration range (Dicke et al. 1990, de Boer and Dicke, 2002).

Methyl salicylate

To test at which concentration the predatory mites were significantly attracted towards the compound methyl salicylate after processing, 5 µg of methyl salicylate was thermally transferred into a 3 L bag. The maximum concentration of methyl salicylate (100 % recovery) in the bag would be 1.67 µg/l. However, the methyl salicylate concentration in the flow out of the bag was expected to be about 40 % (see recovery section of method evaluation experiments, Figure 7) of the amount inside the bag, which amounts to approximately 0.67 µg/l. The flow out of the bag (50 ml/min) was mixed with the make-up airflow of 4 l/min, which gives a calculated methyl salicylate concentration of 8.3 ng/l in the air flow going towards the olfactometer. In both replicate olfactometer tests more predators chose for the airstream with methyl salicylate. This was only statistically significant in one of the tests. However, because the results from the two replicates did not differ significantly, the data were pooled and the total response shows a significant attraction ($P = 0.013$) of the predatory mites towards this concentration of methyl salicylate (Figure 10). When 7.5 µg of methyl salicylate was transferred into the bag, a concentration of 2.5 µg/l was obtained in the bag. The actual methyl salicylate concentration in the flow out of the bag was around 1.0 µg/l and the calculated concentration in the flow towards the olfactometer around 12.3 ng/l. A significant attraction of the predatory mites towards methyl salicylate was obtained for both replicate olfactometer experiments as well as for the combination of the two replicates ($P = 0.8 \times 10^{-5}$) (Figure 10).

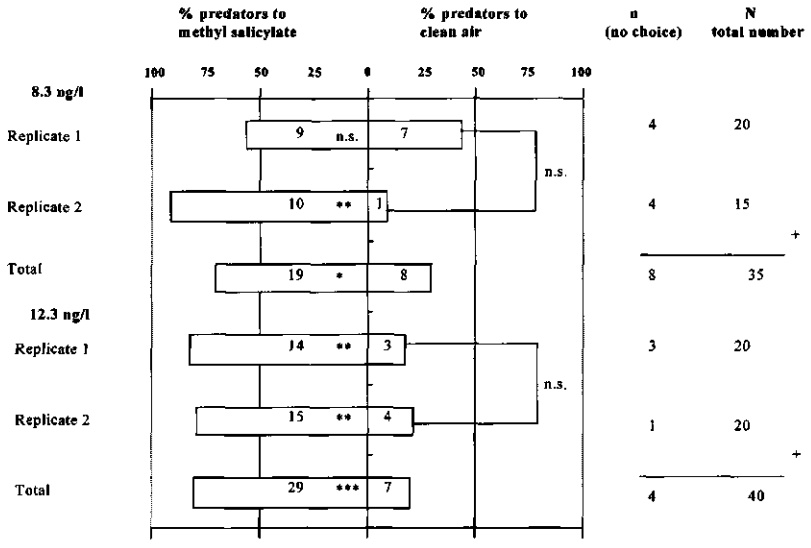

Figure 10: Methyl salicylate was tested versus clean air in an olfactometer in two replicate experiments. A methyl salicylate solution in TBME was injected into the fractionation system and was transferred via thermal desorption into the Teflon bag. In two replicate experiments 8.3 ng/l of methyl salicylate (5 µg in the 3 L bag) and in two other replicate experiments 12.3 ng/l (7.5 µg in the 3 L bag) were tested in the olfactometer. The response of adult females of the predatory mite *P. persimilis* is depicted. Statistics: one-sided binomial test and a 2x2 contingency table test (*** = $P \leq 0.001$, ** = $0.01 \geq P > 0.001$, * = $0.05 \geq P > 0.01$, n.s. = $P > 0.05$)

(3E)-4,8-dimethyl-1,3,7-nonatriene

To test at which concentration the predatory mites were significantly attracted towards the compound (3*E*)-4,8-dimethyl-1,3,7-nonatriene after processing, 20 µg of (3*E*)-4,8-dimethyl-1,3,7-nonatriene was thermally transferred into a 3 L bag. In one experiment a significant attraction of the predatory mites towards the odour source was found. When the total number of predatory mites was regarded, a significant attraction ($P = 0.044$) was found (Figure 11).

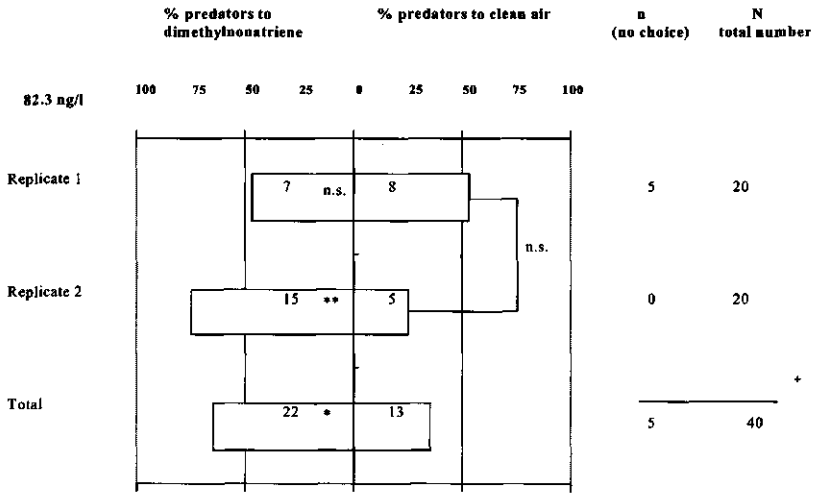

Figure 11: The olfactory response of the predatory mite P. persimilis was tested for (3E)-4,8-dimethyl-1,3,7-nonatriene versus clean air. In two replicate olfactometer experiments 20 µg (3E)-4,8-dimethyl-1,3,7-nonatriene was transferred into the bag (3 L) and an air flow with 82.3 ng/l (3E)-4,8-dimethyl-1,3,7-nonatriene went to the olfactometer. Statistics: binomial test and a 2x2 contingency table test (** = $0.01 \geq P > 0.001$, * = $0.05 \geq P > 0.01$, n.s. = $P > 0.05$)

The calculated maximum concentration of this compound in the flow going out of the bag would be 6.67 µg/l when 100 % recovery was assumed. This would mean that a concentration of 82.3 ng/l (3*E*)-4,8-dimethyl-1,3,7-nonatriene was present in the flow going towards the olfactometer.

Discussion

Method validation

Existing isolation and identification techniques have shown some problems with the extraction and chemical analysis of the active components in complex mixtures. For example, the headspace of spider mite-infested gerbera leaves consists of more than one hundred components, which makes it complicated to identify the volatile synomones that attract the predatory mites (Krips et al., 2001). The fractionation method that is described in this chapter has been developed to deal more effectively with complex mixtures or with synergistic compounds. With the new fractionation method one or more components could be selectively removed out of the blend, while the ratios of the remaining components were kept similar to the original blend. Another advantage is that no solvent or paraffin oil is introduced into the bioassay that can disturb the insect's response towards the infochemicals. With the method developed here, one or more compounds can selectively be taken out of the total mixture (see Figure 8). Subsequently, the volatile mixture can be tested for bioactivity. However, when the volatile blend contains many components, a lot of work needs to be carried out to find the active compounds. To circumvent numerous experiments Turlings and Fritzsche (1999) described a method that tested the attractiveness of various recombined fractions of volatiles from maize seedlings damaged by *Spodoptera* larvae. In this method subtraction was also performed. In each recombination of fractions, one of the four fractions was left out. First, the recombined fractions were individually tested on their attractiveness. Subsequently the recombined fractions were tested against each other in a multiple choice test. In the next multiple choice test one of the investigated mixtures was again reduced by one non-bioactive fraction and tested versus the other recombined fractions. This subtractive method in combination with multiple choice tests is straightforward to find the right synergistic combinations of bioactive fractions. Fewer trials will

be needed to find the bioactive fractions when a multiple choice test is used in comparison to a two choice test. However, a two-choice test is a more reliable bioassay to test insect responses than a multiple choice test.

Recoveries of reference compounds

The method described in this chapter was validated with respect to the recovery of different test compounds for all the separate steps of the fractionation method as well as the overall recoveries. Relatively low recoveries for methyl salicylate, (*E*)-nerolidol and (*E,E*)-farnesol were found. Methyl salicylate contains a phenolic group that could possibly interact with active sites present in the system. (*E*)-nerolidol and (*E,E*)-farnesol have very high boiling points (> 300 °C). Possibly, due to the high boiling points compounds can partially condense in the Teflon bag.

Biological activity after processing

The results depicted in Figure 9 show that the headspace of spider mite-infested lima bean volatiles are still active towards the predatory mites after processing. Furthermore, Figures 10 and 11 show that predatory mites were significantly attracted to low concentrations of methyl salicylate (around 0.01 µg/l). Predatory mites also showed significant attraction to 0.1 µg/l (3*E*)-4,8-Dimethyl-1,3,7-nonatriene. In 1990, Dicke et al. found a significant attraction of predatory mites towards both methyl salicylate (20 µg dissolved in 0.1 ml dichloromethane) and (3*E*)-4,8-dimethyl-1,3,7-nonatriene (20 µg dissolved in 0.1 ml paraffin oil). De Boer and Dicke (2002) tested methyl salicylate applied onto filter paper in the olfactometer recently and found that predatory mites (*P. persimilis*) were attracted to concentrations ranging from 0.2 to 20 µg dissolved in 0.1 ml hexane. In their experiments the actual concentration of methyl salicylate in the airflow depended on the evaporation rate, while in the method proposed in this chapter there is a constant concentration because there is no evaporation step. The

concentration ranges cannot be exactly compared. However, when a linear evaporation rate is assumed within the five minutes that the experiment is carried out, the concentration of de Boer and Dicke (2002) leads to a concentration range of 0.01-1 μg/l in the air stream. This is consistent with the results that were found with the method tested in this chapter.

Conclusion

The method that is described in this chapter is a sophisticated tool to manipulate volatile mixtures. The innovation is that one or more components can be selectively removed from the blend, while the ratios of the remaining components are kept similar to the original blend. Another advantage of this method is that no solvent is introduced into the olfactometer that may interfere with the reaction of the predatory mites. Although for most compounds good recoveries were obtained, the compounds with a high boiling point or a phenolic group are discriminated. This discrimination may be caused by interaction of active sites with methyl salicylate and by condensation that can take place in the Teflon bag due to the high boiling points of (*E*)-nerolidol and (*E,E*)-farnesol. By removing non-active fractions, additive or synergistic effects can be found more easily. From the results that predatory mites are attracted at a concentration level of 0.01 μg/l to methyl salicylate and at a level of 0.1 μg/l to (3*E*)-4,8-dimethyl-1,3,7-nonatriene can be concluded that the described method can be used to screen low quantities of attractive compounds and synergists in a systematical way.

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Discussion

Main questions

Defensive strategies of plants against herbivores are subject to evolutionary changes. Plants have developed two different strategies to defend themselves, namely by direct and indirect defences. In this thesis both types of plant defence have been investigated. As model herbivore and carnivore the spider mite *Tetranychus urticae* and the predatory mite *Phytoseiulus persimilis* were used respectively. The main questions that were emphasised in this thesis are:

- What is the variation in direct defence among various plant species?
- Do the investigated plant species also use indirect defence?
- Are novel compounds present in the headspace of spider mite-infested leaves compared to mechanically damaged leaves?
- Is the hypothesis that plant species with a strong direct defence have a lower investment in indirect defence and vice versa correct? Does this hypothesis also apply at the level of plant family?
- What are the important bioactive compounds in the volatile blends of spider mite-infested plant leaves?
- Are novel biosynthetic pathways induced in response to spider mite-infestation?
- Is the new fractionation method that leads to the biological identification of bioactive compounds, better and more efficient than other frequently used methods?

These questions will be addressed in the discussion below.

Variation in direct defence

The results on spider mite acceptance of different host plants (Chapter 2) show once more that *T. urticae* accepts a broad range of host plants to feed on. This was also suggested by Yano et al. (1998), who investigated several wild plant

species and showed that spider mites differentially accepted them. They also showed that spider mite fecundity is a measure of host plant suitability. In Chapter 2 the degree of spider mite acceptance of different host plants was used as a measure of the constitutive direct defence of a plant species. However, besides toxic or deterrent secondary metabolites, the degree of spider mite acceptance of a host plant can also be affected by other factors; such as the nutritional value of a plant, its physical characteristics and local and / or systemic induction of secondary metabolites. Also the probability that the spider mite encounters carnivorous enemies that might be present on a plant can affect the degree of spider mite acceptance (Schoonhoven et al., 1998). Therefore, more research needs to be carried out to investigate whether toxic or deterrent secondary metabolites are the most important factors to affect the degree of spider mite acceptance. Moreover, secondary metabolites that have a strong deterrent or toxic effect on the spider mite *T. urticae* can be determined and related to a plant's direct defence. Baldwin (1999) showed that after attack by generalist herbivores, tobacco plants invest in the production of nicotine type alkaloids that are very toxic to herbivores. Nicotine synthesis takes place in the roots and increases the nicotine concentrations in the whole plant (Baldwin, 1995, Baldwin et al., 1997). Although *Nicotiana tabacum* (tobacco) was expected to have a strong direct defence, the plant did not deter the spider mites from its leaf discs. This could be explained by the fact that leaf discs are used instead of intact plant species and, therefore, induction of alkaloids might not take place or only in smaller amounts.

Do plants invest in indirect defence?

Inducible defences can benefit a plant, since they are only switched on when the plant is under attack by herbivores or pathogens. In Chapter 3 all *T. urticae*-infested plant species showed a significant attraction of *P. persimilis* towards their spider mite-infested leaves when tested versus non-damaged leaves of the same plant species. This shows that most plants invest in indirect defence. Van

der Meijden and Klinkhamer (2000) stated that especially for tritrophic interactions in which parasitoids are involved the benefit for the plant can be questioned, because parasitoids do not always reduce the fitness loss of an individual plant. However, there are examples when plants show an increase in their seed production after the caterpillars feeding on their leaves had been parasitised (van Loon et al. 2000; Fritzsche-Hoballah and Turlings, 2001). For plant-herbivore-predator interactions an increase in plant fitness is more likely, because the predator will reduce the level of herbivory by killing their prey.

Do plant species emit novel compounds after a spider mite attack?

Through the production of novel compounds the spider mite-infested plant can emit a blend that is qualitatively different from that of mechanically damaged leaves (Dicke, 1994; Dicke et al., 1990; Turlings et al. 1990, 1995; Takabayashi et al., 1994). Many of the investigated plant species produce novel compounds after herbivore-infestation, which indicates that plants invest in a more sophisticated form of indirect defence (Chapter 4). Only spider mite-infested eggplant and tobacco leaves showed mostly quantitative differences compared to clean and mechanically damaged leaves. They only produced minor quantities of novel volatiles. However, these minor compounds can be of great importance. For example, *Cotesia rubecula* is a specialist parasitoid with *Pieris rapae* caterpillars as host. This parasitoid can distinguish between *P. brassicae*-infested and *P. rapae*-infested leaves of Brussels sprouts plants, whereas there are only small differences when the two headspace blends are compared (Smid et al., 2002). Additionally, only quantitative differences have been recorded when the headspace from apple foliage infested with the spider mite *T. urticae* was compared to that from apple foliage infested with the spider mite *P. ulmi*. Nevertheless, the predatory mite *P. persimilis* distinguished between the two odour sources and preferred the headspace of its preferred prey species *T. urticae* (Takabayashi et al., 1991). This shows that carnivorous arthropods may use quantitative differences to discriminate. Alternatively, it may be possible that

minor compounds that are present below the detection limit trigger the response of the predatory mite. Vet and Dicke (1992) suggest that the production of large amounts of novel compounds by a spider mite-infested plant can lead to a better detectable and reliable odour source for carnivorous enemies of the herbivores.

Direct versus indirect defence

In Chapters 2, 3 and 4 the hypothesis was tested that direct and indirect defences are negatively correlated. However, most plant species that were investigated showed a strong sophisticated form of indirect defence with the production of novel compounds even though some of them had a strong direct defence. This indicates that the hypothesis should be rejected, and that both direct and indirect defences can be used by plants to defend themselves against herbivores. The fact that not all plant species have evolved to contain high concentrations of toxic secondary metabolites in their leaves might be explained by variation in generalist and specialist herbivores. Van der Meijden (1996) presented a model that relates plant fitness to the concentration of toxic or deterrent secondary metabolites in a plant. This model assumes that at low levels of secondary metabolites no feeding of specialist herbivores takes place, while at high levels no feeding of generalist herbivores occurs. This leads to an optimum in plant fitness at medium concentration levels of secondary metabolites. Besides herbivore attack, a plant also has to defend itself against fungal, bacterial or viral pathogen attack, which may be done through induced defences. All these different forms of plant defence can lead to considerable costs in terms of fitness for a plant (Baldwin, 1999). Moreover, there may be trade-offs in plant defences against herbivores and pathogens (e.g. Thaler et al 1999). However, the question whether different defence strategies of a plant dealing with herbivores and pathogens can influence each other either positively or negatively still needs to be addressed.

Bioactive compounds

An important question to address is which compounds play a role in the tritrophic interaction of *T. urticae*, *P. persimilis* and several host plants. Additionally, the question needs to be answered whether these compounds are dominantly present in the blend or that minor compounds can also contribute to the attractiveness of a blend. In most of the plant species investigated in Chapter 4 novel compounds were emitted after spider mite-infestation. A few compounds that were known to attract predatory mites, such as methyl salicylate and three terpenes (see Dicke et al. 1990) were dominantly present in some of the spider mite-infested blends of the investigated plant species. Methyl salicylate was dominantly present in six of the investigated plant species and less dominantly in two plant species. Terpenoids such as linalool, (*E*)-β-ocimene and (3*E*)-4,8-dimethyl-1,3,7-nonatriene were dominantly present in respectively one, two and three of the investigated plant species. The presence of methyl salicylate in many of the investigated plant species indicates that it might be an important compound that is specifically induced by the spider mite *T. urticae*. This idea is strengthened by the fact that the compound was not found in the volatile blend emitted by lima bean leaves infested with caterpillars of *Spodoptera exigua* or *Mythimna separata* (Ozawa et al., 2000). Methyl salicylate was also not produced by maize and cowpea plants treated with regurgitant of the caterpillar *S. littoralis* (Fritzsche-Hoballah et al., 2002). However, *Arabidopsis thaliana* leaves infested with *Pieris rapae* caterpillars emitted methyl salicylate as a major compound in the blend (van Poecke et al., 2001). It can be concluded that although methyl salicylate is present in most of the spider mite-infested plant species, it does not necessarily indicate spider mite-damage. From these results it can be derived that methyl salicylate is not the only compound that is involved in the tritrophic interaction under investigation. Other terpenoid compounds than linalool, (*E*)-β-ocimene and (3*E*)-4,8-dimethyl-1,3,7-nonatriene or other non-terpenoid compounds that were emitted by the investigated plant species may lead to predatory mite attraction as well, although their attractiveness still has to be

investigated. Van Poecke (2002) showed that in the headspace of NahG-type transgenic *A. thaliana* plants no methyl salicylate and less terpenes were present than in wild-type plants. This type of *A. thaliana* cannot accumulate salicylic acid due to the expression of the bacterial salicylate hydroxylase gene NahG (Delaney et al., 1994). Therefore, it will be interesting to see if NahG plants attract the predatory mite *P. persimilis* after being infested with the spider mite *T. urticae*.

Novel pathways

Most of the plants seem to actively attract predators or parasitoids with the production of novel compounds, compared to mechanically damaged plant leaves. This represents the induction of novel biosynthetic pathways (Bouwmeester et al., 1999; Dicke, 1999; Paré and Tumlinson, 1997). Moreover, van Poecke et al. (2001) suggested that gene expression was induced by *P. rapae* caterpillars when they were feeding on *A. thaliana*. Methyl salicylate, which is a key compound in the shikimic acid-pathway (Karban and Baldwin, 1997; Malamy et al., 1996; Metraux et al., 1990; Ryals et al., 1995) can play a role in the induction of indirect defences in a large number of the investigated plant species. This pathway is induced when a plant increases its systemic acquired resistance (SAR) against pathogens by salicylic acid that provides a signal for the expression of pathogenesis-related (PR) proteins (Raskin, 1992; Ryals et al., 1995). Also some terpenoids are released as novel compounds from most of the investigated plant leaves. These compounds are produced via biosynthetic pathways that are mostly triggered via the octadecanoid pathway. Jasmonic acid is one of the plant hormones active in this signal transduction pathway (Farmer and Ryan, 1992; Wasternack and Parthier, 1997). The octadecanoid pathway is induced when a plant increases its direct defence against both pathogens and herbivores. Besides direct defence, indirect defence is induced by the octadecanoic pathway as well. When lima bean or gerbera plants were treated with jasmonic acid, a volatile blend was emitted with a profile that is

qualitatively almost similar to a spider mite-induced blend for the same plant species. The jasmonic acid-induced blend of both plant species attracted the predatory mite *P. persimilis* (Dicke et al. 1999, Gols et al. 1999). One major difference is that methyl salicylate was not emitted. It can be concluded that the induction of both shikimic acid- and octadecanoid pathways can contribute to the level of direct and indirect defence of a plant species.

Fractionation method

To obtain more knowledge on herbivore-infested plant species that can emit a reliable odour blend to attract predatory mites, biological identification of synomones in tritrophic interactions is important. The fractionation system described in this thesis is potentially a sophisticated tool to identify these compounds. However, more experiments need to be carried out to show the efficiency of this method when complete plant mixtures are tested. For example, a total plant mixture can be tested against the processed plant mixture minus one or more of the compounds that are supposed or known to show bioactivity. When the predatory mite significantly prefers the intact plant mixture, the compound that was eliminated from the plant mixture is a contributor to the bioactivity of the whole mixture. Another way to identify the bioactive compounds in a volatile blend from spider mite-damaged leaves is to test the processed blend minus one or more of compounds that are expected to contribute to the plants bioactivity, versus the processed blend of mechanically damaged leaves. When the fractionated blend of spider mite-damaged leaves is similar or less attractive towards the predatory mites compared to the processed mechanically damaged leaves, the bioactive compounds can be identified more easily. After more validation and optimisation the fractionation system can contribute to the knowledge on bioactive compounds in tritrophic interactions.

In summary, plant species use both direct and indirect defence to defend themselves against the spider mite *T. urticae*. Most investigated plant species used a sophisticated form of indirect defence by the production of novel compounds after spider mite-infestation. However, in future studies more investigations need to be carried out to identify which compounds play a role in the interaction with the predatory mite *P. persimilis*. Another point of attention is whether bioactive compounds are dominantly present in the headspace emitted by the plant or not. The developed fractionation method can be useful to determine bioactive compounds in tritrophic interactions.

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Summary

The spider mite *Tetranychus urticae* Koch is a serious pest in field crops, glasshouse vegetables and fruit crops. It is a generalist herbivore with several hundreds of host plant species. *Phytoseiulus persimilis* Athias-Henriot is one of its natural enemies. Investigations of the tritrophic system of plant, *T. urticae* and *P. persimilis* will contribute to a better knowledge about the direct and indirect defence defensive strategies of plant species.

Host plant acceptance by the spider mite *T. urticae*, as a measure of the plant's direct defence, was investigated for eleven plant species. The degree to which the spider mites accepted a plant was expected to depend on differences in nutritive and toxic constituents among plant species. At the level of plant species, a large variation in the degree of acceptance by *T. urticae* was found. Except for ginkgo (*Ginkgo biloba*) most plants were accepted or well accepted by the spider mites. At the level of plant family, four plant species from the Fabaceae were compared to four plant species from the Solanaceae. It was shown that all species from the Fabaceae were accepted by the spider mites for feeding, while plant species from the Solanaceae varied in spider mite acceptance from well accepted (tobacco: *Nicotiana tabacum*) to poorly accepted (sweet pepper: *Capsicum annuum*).

Some of the plant species that had already been investigated with respect to spider mite acceptance were tested for their degree of indirect defence. After spider mite-infestation the plants attracted the predatory mite *P. persimilis*. The results showed that plants from all species significantly attracted the predatory mites when infested by spider mites. Experience with the spider mite-infested leaves of the investigated plant species did not affect the predatory mite's choice. Based on the results that spider mites did not survive on ginkgo leaves, these leaves were treated with jasmonic acid to induce a mimic of a spider mite-

induced volatile blend. The predatory mites were slightly attracted to the induced volatile blend of jasmonic acid treated leaves. In summary, plants do invest in indirect defence after being attacked by spider mites, even when some plants have already a strong direct defence.

Subsequently, it was investigated to what degree spider mite-infestation of plants from all species resulted in the emission of novel compounds that were not emitted by undamaged or mechanically damaged plants of the same species. Therefore, the volatiles emitted by *T. urticae*-infested leaves were analysed and compared to volatiles emitted by clean and mechanically damaged leaves of the same plant species. Almost all of the investigated plant species produced novel compounds that dominated the volatile blend after spider mite infestation, such as methyl salicylate, terpenes, oximes or nitriles. However, spider mite-infested eggplant and tobacco emitted only a few novel compounds and in small amounts. Methyl salicylate was found as dominant compound in six of the investigated plant species and as a less dominant compound in two plant species. However, it was concluded that methyl salicylate alone does not necessarily indicate spider mite-damage of the plant.

In the introduction (Chapter 1) a hypothesis was postulated that plant species with a weak direct defence would invest in the production of novel compounds after spider mite-infestation, in contrast to plant species that possessed a strong direct defence. However, although plant species that have a weak direct defence can use indirect defence to defend themselves, they do not always emit novel compounds. At the level of plant family qualitative differences in volatile blends from spider mite-infested leaves compared to mechanically-damaged leaves were more prominently found in the Fabaceae than in the Solanaceae.

A fractionation method was developed for identification of the biologically active compounds in mixtures of volatile compounds (or volatile mixtures),

which is more selective and efficient than conventional techniques such as comparison of volatile profiles or the use of synthetic mixtures. With this method bioactive compounds that mediate interactions within and among species can be determined more quickly. First, separation of the volatile mixture was carried out in a gas chromatograph. This made it possible to selectively remove compounds from the mixture. Before the volatiles were tested on bioactivity in an olfactometer, the compounds were revolatilised by thermal desorption and stored in a Teflon bag. Subsequently, the Teflon bag was pressurised and a continuous flow of volatiles was led to the olfactometer.

Validation of the method showed that most of the investigated compounds that varied in boiling points and in chemical nature showed a high recovery (80-100 %). Only compounds that had a relatively high boiling point (> 300 °C) or contained a phenolic group showed low recoveries (30-50 %). The biological activity of the volatiles emitted by *T. urticae*-infested lima bean leaves (*Phaseolus lunatus*) and the compounds methyl salicylate and (3*E*)-4,8-dimethyl-1,3,7-nonatriene were successfully tested after being processed with the method. An advantage of the method is that volatile blends can selectively be manipulated. Besides, solvent introduction into the bioassay can be circumvented. After processing and revolatilization of the mixture, a known concentration of the volatiles can be led to the bioassay. In potential, the method can be useful to determine biologically active compounds from complex mixtures in the future.

In conclusion, this thesis presents a comparative analysis of direct and indirect defence of different plant species against the spider mite *T. urticae*. Qualitative versus quantitative differences are found for volatile blends that are emitted from spider mite-infested plant leaves compared to clean or mechanically damaged leaves. Novel compounds that dominate the blends from spider mite-infested plants are discussed for the investigated plant species. The newly developed fractionation method is shown to be a valuable and efficient method to obtain more knowledge on bioactive compounds in complex mixtures of plant volatiles.

Samenvatting

Planten hebben niet alleen interacties met hun belagers maar ook met de natuurlijke vijanden van hun belagers. We spreken dan van een tritroof systeem; dat wil zeggen dat drie verschillende niveaus uit een voedselketen onderlinge interacties hebben. In dit proefschrift is een tritrofe interactie onderzocht waar de spintmijt *Tetranychus urticae*, de roofmijt *Phytoseiulus persimilis* en verscheidene waardplanten deel van uitmaken. De spintmijt *T. urticae* is een alleseter en heeft een paar honderd soorten waardplanten. Hij kan een enorme plaag veroorzaken in akkerbouwgewassen, fruitbomen en glasgroenten. De roofmijt *P. persimilis* is één van zijn belangrijkste natuurlijke vijanden.

Planten kunnen zich op verschillende manieren verdedigen tegen een herbivoor. Als er sprake is van het gebruik van toxische of smaakvergallende stoffen dan noemen we dit directe verdediging. Indien de plant gebruik maakt van de aantrekking van de natuurlijke vijanden van de herbivoor (carnivoren) met behulp van vluchtige stoffen dan noemen we dit indirecte verdediging. Onderzoek naar de directe en indirecte verdedigingsstrategieën van verscheidene plantensoorten zal bijdragen tot het verkrijgen van meer kennis in de voedselketen plant – spintmijt – roofmijt.

Als eerste is de acceptatie van elf waardplanten door de spintmijt *T. urticae* onderzocht. Deze waardplantacceptatie wordt gebruikt als maat voor de directe verdediging van deze plantensoorten. Verwacht wordt dat de mate waarin de spintmijt de waardplant accepteert, afhankelijk is van de voedingswaarde van de plant en het voorkomen van toxische of smaakvergallende stoffen in een plant. De proeven laten een grote variatie zien in de mate waarin de spintmijt de planten accepteert. De meeste planten worden goed tot zeer goed geaccepteerd. Alleen ginkgo (*Ginkgo biloba*) vormt hierop een uitzondering.

Naast de verschillen tussen waardplantsoorten zijn twee plantenfamilies de Fabaceae (Vlinderbloemigen) en de Solanaceae (Nachtschadeachtigen) met elkaar vergeleken, van beide families zijn vier plantensoorten betrokken bij het onderzoek. Uit deze vergelijking blijkt dat alle soorten behorend tot de Fabaceae goed geaccepteerd worden door de spintmijt, maar de Solanaceae soorten variëren in de mate waarin ze door de spintmijt geaccepteerd worden van goed (tabak: *Nicotiana tabacum*) tot slecht (paprika: *Capsicum annuum*).

Vervolgens is de indirecte verdediging van de plantensoorten onderzocht en hiervoor zijn dezelfde plantensoorten gebruikt. In dit experiment worden bladeren die door spintmijten zijn aangetast naast onbeschadigde bladeren aangeboden aan de roofmijt *P. persimilis* in een olfactometer als een keuzetoets-opstelling. Er is nagegaan of bladeren met spintmijt aantrekkelijker zijn voor de roofmijt dan bladeren zonder spintmijt. De resultaten laten zien dat de door spintmijt aangetaste bladeren van alle plantensoorten de roofmijt significant aantrekken. Eerdere experimenten uit de literatuur laten zien dat de spintmijten zelf niet aantrekkelijk zijn voor roofmijten. Hieruit kan worden geconcludeerd dat de stoffen die uit de plant vrijkomen, de roofmijten aantrekken. Eerder opgedane ervaring van de roofmijt met door spintmijt aangetaste bladeren van de plantensoort die wordt getoetst vertoont geen invloed op de mate van aantrekking van de roofmijt. Aangezien de spintmijten het niet overleefden wanneer ze de bladeren van de ginkgoboom als voedsel aangeboden kregen, is het ginkgoblad behandeld met jasmonzuur, om op deze wijze een vergelijkbaar mengsel van vluchtige plantenstoffen te produceren als spintmijt besmette bladeren mogelijk zouden kunnen produceren. Uit deze proef komt naar voren dat roofmijten in een geringe mate aangetrokken worden tot de met jasmonzuur behandelde bladeren. Er kan geconcludeerd worden dat alle onderzochte plantensoorten na besmetting met spintmijt investeren in indirecte verdediging, zelfs indien ze al een sterke directe verdediging hebben.

Toen vastgesteld was dat alle onderzochte planten na aantasting door spintmijt aantrekkelijk waren voor roofmijten, is onderzocht welke verbindingen deze planten produceren. Tevens is gekeken in welke mate de geproduceerde mengsels van vluchtige verbindingen nieuwe stoffen bevatten in vergelijking met mengsels van niet-beschadigde of mechanisch beschadigde bladeren. De analyses van de vluchtige mengsels die door de met spintmijt aangetaste bladeren worden uitgestoten tonen aan dat alle onderzochte planten nieuwe verbindingen produceren. Een aantal van deze nieuw geproduceerde verbindingen zijn dominant aanwezig in het mengsel, zoals methylsalicylaat en verscheidene terpenen, oximen en nitrillen. Echter, aubergineplanten en tabaksplanten produceren na spintmijt aantasting slechts enkele nieuwe componenten in kleine hoeveelheden. Methylsalicylaat is in zes van de onderzochte plantensoorten gevonden als de dominant aanwezige verbinding in het mengsel, in twee andere plantensoorten is methylsalicylaat minder dominant aanwezig. Hoewel is aangetoond dat methylsalicylaat roofmijten aantrekt, is deze verbinding op zichzelf niet specifiek genoeg om te kunnen dienen als een indicator voor spintmijt aantasting van planten.

In hoofdstuk 1 wordt de aanname gemaakt dat plantensoorten met een zwakke directe verdediging zouden investeren in de productie van nieuwe verbindingen, terwijl planten met een sterke directe verdediging hierin niet zouden hoeven te investeren. Uit de resultaten komt naar voren dat plantensoorten met een zwakke directe verdediging inderdaad indirecte verdediging gebruiken om roofmijten aan te trekken, maar dat ze hiervoor niet altijd nieuwe verbindingen produceren. Voor twee plantenfamilies zijn de vluchtige mengsels die geproduceerd worden door spintmijt aangetaste bladeren vergeleken met mengsels die geproduceerd worden door mechanisch beschadigde bladeren. Hieruit komt naar voren dat deze kwalitatieve verschillen in vluchtige mengsels afkomstig van door spintmijt aangetaste bladeren meer prominent worden aangetroffen in planten van de Fabaceae dan in die van de Solanaceae.

Om biologisch actieve verbindingen in mengsels van vluchtige stoffen beter te kunnen opsporen en identificeren is een nieuwe fractioneringsmethode ontwikkeld. Deze fractioneringsmethode is selectiever en efficiënter in het opsporen van biologisch actieve verbindingen dan de gangbare technieken, waarbij gebruik wordt gemaakt van het vergelijken van profielen van vluchtige mengsels, of waarbij mengsels van biologisch actieve mengsels nagemaakt worden met behulp van synthetische verbindingen. De eerste stap in deze fractioneringsmethode is het scheiden van stoffen van een biologisch actief mengsel met een gaschromatograaf. De gaschromatografische scheiding maakt het mogelijk om selectief verbindingen uit het mengsel te verwijderen. Vervolgens worden de verbindingen opgevangen in een buis gevuld met adsorptiemateriaal (Tenax) en daarna weer vluchtig gemaakt door middel van verhitting (thermodesorptie) en opgevangen in een Teflon zak. Om de verbindingen te kunnen testen op hun biologische activiteit met behulp van een olfactometer, wordt de Teflon zak onder druk gezet zodat er een continue zwakke stroom van vluchtige stoffen ontstaat. Deze zwakke stroom wordt vervolgens meegenomen in een luchtstroom en naar de olfactometer geleid. In de olfactometer wordt het hele mengsel (of het overgebleven mengsel) getoetst om vast te kunnen stellen of het mengsel nog biologisch actief is.

De werking van de methode is gecontroleerd met behulp van vluchtige stoffen die variëren in kookpunt en in hun chemische- en fysische eigenschappen. De stoffen zijn na het proces opnieuw opgevangen om te kijken hoeveel procent van deze stoffen na het hele proces nog overgebleven zijn. De meeste stoffen die zijn getest worden in een hoge opbrengst van 80 en 100 % teruggewonnen. Slechts enkele stoffen met een relatief hoog kookpunt (> 300 °C) of met een fenolgroep, geven lage opbrengsten (30–50 %). De biologische activiteit van de stoffen die geproduceerd worden door spintmijt aangetaste bladeren van de limaboon (*Phaseolus lunatus*) en de verbindingen methylsalicylaat en (3*E*)-4,8-dimethyl-1,3,7-nonatrieen zijn met succes getest op hun aantrekkelijkheid voor roofmijten. Een voordeel van deze methode is dat mengsels van vluchtige

stoffen selectief gemanipuleerd kunnen worden. Een tweede voordeel is dat met deze methode geen oplosmiddel in de biotoets wordt geïntroduceerd. Door het mengsel met deze methode te bewerken en opnieuw vluchtig te maken is het mogelijk om een bekende en realistische concentratie van de verbindingen in de biotoets te brengen. Dit maakt deze fractioneringsmethode tot een handige en snelle werkwijze om biologisch actieve verbindingen in complexe mengsels op te kunnen sporen en vervolgens te kunnen identificeren.

Curriculum Vitae

Cindy Elise Marieke van den Boom werd geboren op 17 februari 1973 te Valkenswaard. In 1991 behaalde ze het Atheneum diploma aan het Anton van Duinkerken College te Veldhoven. Aansluitend studeerde ze Scheikundige Technologie aan de Technische Universiteit Eindhoven. Tijdens deze studie deed ze een afstudeervak analytische chemie aan de Universiteit 'Virginia Tech' in Virginia, de Verenigde Staten. Ze behaalde haar ingenieursdiploma in december 1996 om enkele maanden later aan de Wageningen Universiteit een promotieonderzoek te beginnen bij de leerstoelgroep Organische Chemie, onder leiding van dr. T.A. van Beek en prof. dr. Ae. de Groot tezamen met de leerstoelgroep Entomologie, onder leiding van prof. dr. M. Dicke. Het hiervoor liggende proefschrift over plantenstrategieën in een tritroof systeem van spintmijt, roofmijt en diverse plantensoorten is hiervan het resultaat. Sinds juli 2001 is ze werkzaam als projectleider bij de afdeling Agrofood bij Stichting Milieukeur te Den Haag.

List of publications

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Omslag: Judith Brüggenwirth